Biological and Genetic Studies on the E2 Glycoprotein of Ross River Virus

by

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Statement

All the work reported in this thesis was done by myself with the exception of the statistical analyses in Chapters 3 and 4 which were performed by Mr. Ross Cunningham, Department of Statistics, The Faculties, Australian National University.

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Abstract

The results presented in this thesis have focussed attention on the biological role of the E2 glycoprotein of RRV and the viral functions associated with neutralization epitopes. Previous work had defined three such epitopes on E2 of RRV at amino acids 216 (epitope a), 232, 234 (epitope b1) and 246, 248, 251 (epitope b2) by the selection of monoclonal antibody (mAb) escape mutants. An additional epitope (c) had been defined by competitive binding assays. It was postulated that these epitopes formed part of a major neutralization domain on E2 (Vrati *et al.*,1988).

This hypothesis was examined by raising antisera to the parental RRV T48 in mice and comparing the neutralization mAb resistant variants by this antisera with T48. A triple mAb resistant variant of T48 altered in epitopes a, b1 and b2 (Tv161) was neutralized three to eight fold less efficiently by hyperimmune sera than T48. Neutralization of variants with changes in single epitopes was not altered. RRV NB5092, which is over 2% diverged from T48 in its nucleotide sequence (Faragher *et al.*,1988) could not be distinguished from T48 using these sera. It was concluded that a significant proportion of the antibodies present in a hyperimmune response to RRV were directed at this group of epitopes and therefore that this region could be a target for a synthetic vaccine.

To explore this further, four synthetic peptides, representing most of the primary amino acid sequence between amino acids 209 and 253, were used to raise antisera in mice. The reactivity of these sera with RRV was examined in a range of assays. Anti-peptide sera bound to RRV very specifically in a direct ELISA. However, these sera did not neutralize RRV nor did they bind to the virus in a capture ELISA. This suggested that the conditions of binding RRV to the plastic surface in a direct ELISA partially denatured the virus exposing regions on E2 that were recognized by anti-

peptide antibodies. However, in native virions these epitopes were not exposed. In support of this neither mAbs nor polyclonal antibodies raised against RRV bound to the peptides in a variety of assays. It was concluded that either the antigenic determinants in this region were discontinuous or, that if continuous sequence loops existed their secondary structure was constrained such that antibodies raised to synthetic peptides did not recognize the loops, or that individual peptides contained insufficient residues to mimic such structures.

It had previously been shown that two variants of T48 with single amino acid alterations in the b2 epitope penetrated faster than T48 in cultured BHK cells (Vrati, 1986). This work was extended here by demonstrating that all changes in the b2 epitope from the T48 sequence gave fast penetration in BHK cells and that fast penetration also occurred in Vero cells. Changes at epitopes a or b1 did not alter penetration rate in either cell line. Examination of the penetration rates of five geographic variants of RRV with or without differences from T48 at epitope b2, indicated that all had fast penetration compared to T48. The fast penetration of PB629 and GG2227 which have only a single amino acid difference from T48 in E2 (Leu67→Ile) indicated that changes elsewhere in the genome, possibly towards the N-terminus of E2, could give fast penetration. The wide range of amino acid substitutions in epitope b2 that produced fast penetration and the fast penetration of the geographic variants suggests that there may be a specific conformation on the surface of T48 virions that produces slow penetration in tissue culture. Changes at epitope b2 could destabilize or alter this conformation leading to fast penetration. The mechanism of the increased rate of penetration is not clear but preliminary evidence suggested that a faster rate of attachment to cells may be involved.

Having partially defined a domain which was involved in both neutralization and cell entry it was of interest to define further regions of the structural glycoproteins of RRV which could be involved with cell recognition and entry. This was approached by limited passaging of RRV NB5092 in avian cells. Variants of RRV NB5092 selected by passaging in CEFs had single, non-conservative amino acid alterations in E2 at either amino acids 4 or 218. Both changes affected neutralization by mAbs; the variants altered at 218 were neutralized less efficiently by mAb T10C9 (epitope a) while the variant altered at amino acid 4 was neutralized less efficiently by mAbs NB3C4 (epitope b1) and T1D11 (epitope c). Variants with the 218 alteration were attenuated for growth in mice while the variant altered at 4 had a pattern of mouse tissue growth somewhat different to NB5092. Thus alterations in neutralization epitopes on E2 were associated with passaging RRV in avian tissue culture and with changes in growth of the passaged virus in mice.

To extend this work on passaging, RRV strains T48 and NB5092 were passaged in human and mosquito cells. Several amino acid changes in E2 were found following passage in human cells. These were at 178 (Arg→Leu) and 119 (Asn→Tyr) for NB5092 passaged in SW13 cells, while in the same cell line, one clone of T48 had a Pro162→Ser alteration. Only one change was found in NB5092 and T48 passaged in the other human cell lines examined; one clone of T48 had an amino acid change at 11 (Ala→Thr) after 10 passages in 293 cells. These changes did not alter neutralization by the available mAbs. Thus passaging RRV in human cells selected for changes at a different set of amino acid positions to passaging in avian cells and did not select changes in defined neutralization epitopes. Passaging RRV in mosquito cells did not select variants altered in their E2 sequence.

Abbreviations

ABTS	2,2-azino bis 3-ethylbenzthiazoline sulphonate
AMD	actinomycin D
BHK	baby hamster kidney
BP	back-passaged
BS	bovine serum
BSA	bovine serum albumin
CBA	competitive binding assays
cDNA	complementary DNA
CEF	chick embryo fibroblast
C-F	Chou-Fasman
CNS	central nervous system
cpe	cytopathic effect
cpm	counts per minute
C-terminal	carboxy-terminal
ddNTP	dideoxynucleoside triphosphate
DI	defective interfering
dNTP	deoxynucleoside triphosphate
DTT	dithiothreitol
EBM	Eagle's basal medium
EDTA	ethylenediaminetetra-acetic acid
EEE	Eastern equine encephalitis virus
ELISA	Enzyme linked immunosorbent assay
EMEM	Eagle's minimal essential medium
EMEM-M	Eagle's minimal essential medium minus methionine
EV	extracellular virus
FCS	foetal calf serum

FIA	Freund's incomplete adjuvant
FMDV	foot-and-mouth disease virus
GMEM	Glasgow minimum essential medium
GOR	Garnier-Osguthorpe-Robson
HBSS	Hank's balanced salt solution
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid
ic	intracerebral
IC/RNA	intracellular RNA
ip	intraperitoneal
kb	kilobase
K-D	Kyte-Doolittle
KLH	keyhole limpet haemocyanin
LD_{50}	lethal dose for 50% of animals
mAb	monoclonal antibody
MAY	Mayora
MBS	m-maleimidobenzoyl-N-hydroxysuccinimide
MI	mock-infected
MID	Middelburg
min	minute
moi	multiplicity of infection
mRNA	messenger RNA
mw	molecular weight
nd	not determined
NDU	Ndumu
np	non-plaque purified
nsP	nonstructural protein
N-terminal	amino-terminal
OD	optical density

ORF	open reading frame
ONN	O'nyong nyong
PBS	phosphate buffered saline
PEG	polyethylene glycol
pfu	plaque forming unit
pi	post-infection
PIPES	piperazine-N-N'-bis(2-ethane sulfonic acid)
poly-A	polyadenylic acid
poly-U	polyuridylic acid
PRNA	plaque reduction neutralization assay
rpm	revolutions per minute
RRV	Ross River virus
sc	subcutaneous
SDS	sodium dodecyl sulphate
SFV	Semliki Forest virus
SIN	Sindbis virus
TCA	Trichloracetic acid
ts	temperature sensitive
VEE	Venezuelan equine encephalitis virus
WEE	western equine encephalitis virus
ws	working stock

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Chapter I

General Introduction

1.1 Alphavirus classification and biology

Ross River virus (RRV) is an Australian alphavirus. The alphaviruses are small, enveloped, positive strand RNA viruses classified in the family *Togaviridae*. There are currently 26 recognized alphaviruses (Westaway *et al.*,1985); six serological complexes have been defined on the basis of their antigenic relationships, primarily in cross-neutralization tests (Calisher *et al.*,1980). These are the Eastern equine encephalitis (EEE), Middelburg (MID), Ndumu (NDU), Semliki Forest (SFV), Venezuelan equine encephalitis (VEE) and Western equine encephalitis (WEE) virus complexes (Table 1.1). The first three complexes contain a single virus type, while the remaining three contain a number of types and subtypes. RRV, the subject of this thesis, is a subtype within the SFV complex.

Alphaviruses are arboviruses maintained in nature by biological transmission between susceptible vertebrate hosts and haematophagous arthropod vectors, usually mosquitoes (Chamberlain, 1980). The basic vertebrate hosts are either wild birds or rodents; man-mosquito cycles also occur with Chikungunya (CHIK), O'nyong nyong (ONN) and, more recently, RRV (Chamberlain, 1980; Marshall and Miles, 1984). Alphaviruses have a world wide distribution in tropical and temperate regions and appear to be maintained in localized ecological niches. This may favour gradual divergence and speciation as viruses adapt to local hosts and vectors (Chamberlain, 1980).

1.2 Alphaviruses and disease

Although alphaviruses are generally regarded as not causing disease symptoms in their natural hosts and vectors the evidence for this is limited (Chamberlain, 1980). In alphavirus infections in animals, humans

Table 1.1

Serological classification of alphaviruses*

Complex	Species	Subtype
Eastern equine encephalitis (EEE)	EEE	
Middelburg (MID)	MID	
Ndumu (NDU)	NDU	
Semliki Forest (SFV)	SFV	
	Chikungunya (CHIK)	CHIK
	Getah (GET)	GET
		Sagiyama (SAG)
		Bebaru (BEB)
		Ross River (RRV)
	Mayaro (MAY)	MAY
		Una
Venezuelan equine encephalitis (VEE)	VEE	VEE
		Everglades (EVE)
		Mucambo (MUC)
		Pixuna (PIX)
		Cabassou (CAB)
Western equine encephalitis (WEE)	WEE	
	Highlands J (HJ)	
	Fort Morgan (FM)	
	Sindbis (SIN)	SIN
		Whataroa (WHA)
		Kyzylagach (KZH)
	Aura	

* From Calisher *et al.* (1980)

and birds two major syndromes are seen (Shope, 1980): encephalitis, associated with infections by EEE, WEE and VEE and arthritis, fever and rash caused by infection with a wide range of alphaviruses such as RRV, CHIK, ONN and Mayoro (MAY). The equine encephalitis viruses occur exclusively in the Americas where they are responsible for outbreaks of, often fatal, disease in man and animals. CHIK and ONN have caused epidemics in Africa involving millions of human cases (Shope, 1980). Vaccination is only used against the equine encephalitides, mainly in horses and pheasants but also in laboratory workers (Ardans, 1982; Eisner and Nusbaum, 1983; Nathanson and Miller, 1982).

There have been four alphaviruses isolated in Australia, these are RRV (Doherty *et al.*,1963a), Sindbis (SIN), Getah (GET) (Doherty *et al.*,1963b), and Barmah Forest virus (BF) (Marshall *et al.*,1982a). Of these RRV is the most frequently associated with disease (Shope, 1980).

1.3 RRV: biology and epidemiology

1.3.1 Association of RRV with epidemic polyarthritis

The prototype T48 strain of RRV was isolated from *Aedes vigilax* mosquitoes collected near Townsville, Queensland, in 1959 (Doherty *et al.*,1963a). Serological evidence indicated that this virus was the likely aetiological agent of the disease epidemic polyarthritis (Doherty *et al.*,1963a; Doherty *et al.*,1964). However, despite many attempts, RRV was not isolated from clinical cases of epidemic polyarthritis until an outbreak which spread through the Pacific islands between 1979-1981. This epidemic involved tens of thousands of people and RRV was readily isolated from patients with epidemic polyarthritis, finally confirming its role in the disease (Aaskov *et al.*,1981a; Marshall and Miles, 1984; Rosen *et al.*,1981; Tesh *et al.*,1981). RRV has subsequently been isolated from clinical cases of epidemic polyarthritis in Australia (Aaskov *et al.*,1985).

The predominant clinical symptom of epidemic polyarthritis is arthralgia, usually of the small joints of the extremities; this may be accompanied by a macropapular rash, usually on the trunk (Mudge and Aaskov, 1983). Other, more non-specific, symptoms reported include paraesthesia, low grade fever, myalgia, chills and mild lymphadenopathy (Marshall and Miles, 1984). Complete recovery is normal although in some cases the course of the disease is prolonged beyond a few weeks (Mudge and Aaskov, 1983).

Symptoms of a disease resembling epidemic polyarthritis were first reported in Australia, in the Murrumbidgee area of New South Wales, in 1928 (Nimmo, 1928; Edwards, 1928). Further outbreaks were recorded in New Guinea, northern Australia and the Solomon islands during the Second World War (Halliday and Horan, 1943; Dowling, 1946). Since then there have been many outbreaks of epidemic polyarthritis in Australia with especially large episodes recorded in the Murray Valley in 1956 (Anderson and French, 1957), 1971 (Seglenieks and Moore, 1974), 1974 and 1984 (Marshall and Miles, 1984; Hawkes *et al.*,1985). In north-eastern Australia annual outbreaks of epidemic polyarthritis occur (Doherty *et al.*,1971; Clarke *et al.*,1973; Doherty, 1974; Aaskov *et al.*,1981b). The disease has been diagnosed in all states of Australia, including Tasmania (Mudge *et al.*,1981) and antibodies to RRV are widespread in man and animals throughout eastern Australia (Doherty *et al.*,1966). Clinical symptoms appear to vary between epidemics (Anderson and French, 1957; Seglenieks and Moore, 1974; Mudge,1977; Aaskov *et al.*,1981b; Rosen *et al.*,1981). In a recent outbreak in New South Wales (Hawkes *et al.*,1985), arthralgia was the most common clinical finding, followed by lethargy, rash and headache. The estimated subclinical to clinical ratios vary widely between epidemics with recent outbreaks of RRV appearing to cause epidemic polyarthritis in a much greater proportion of infections than previously

reported (Hawkes *et al.*,1985). This may relate to the level of viraemia which could reflect the ability of humans to act as hosts during particular epidemics. Taken together with the variation in clinical symptoms, this may reflect strain differences of RRV.

1.3.2 Mosquito vectors of RRV

RRV has been isolated from at least nine species of mosquito, comprising five genera; other species transmit RRV experimentally (Marshall and Miles, 1984; I.D. Marshall, pers. comm.). Most Australian isolates have come from either *Aedes vigilax* or *Culex annulirostris*. In temperate regions *Culex annulirostris* may be more important in maintaining primary cycles than as an epidemic vector because many outbreaks of epidemic polyarthritis occur in autumn when the numbers of this mosquito have decreased (Marshall and Miles, 1984). During the Pacific Island epidemic *Aedes polynesiensis* appears to have been the principal vector, although other species were probably involved (Rosen *et al.*,1981; Marshall and Miles, 1984). Thus RRV is able to utilize a number of different mosquito species as vectors, although some may be more efficient than others (Marshall and Miles, 1984).

1.3.3 Vertebrate hosts of RRV

A number of native and domestic mammals and marsupials have been implicated as potential vertebrate hosts for RRV, but no definitive host has been identified (Doherty *et al.*,1966; Whitehead, 1969; Doherty *et al.*,1971; Gard *et al.*,1973). It is probable that different mammals or marsupials are important in separate enzootic foci. The outbreak of epidemic polyarthritis in the Pacific islands has convincingly demonstrated that humans can act as the principal host of RRV during epidemics (Marshall and Miles, 1984). RRV has been isolated once from birds (Whitehead *et al.*,1968), but serological data (Doherty *et al.*,1966, Gard

et al.,1973; Marshall *et al.*,1982b) and the results of experimental infections of birds (Whitehead, 1969; Marshall and Miles, 1984) suggest that birds have little role in the ecology of RRV.

1.3.4 Geographic variants of RRV

Enzootic variants of RRV appear to exist in regions geographically isolated from each other. Restriction digest analysis of single stranded cDNA synthesized against viral RNA defined three genetic types from 15 RRV geographic isolates (Faragher *et al.*,1985). Isolates of RRV from Nelson Bay in New South Wales (Gard *et al.*,1973) differed in antigenic and biological properties from the prototype T48 strain. A notable difference being the low virulence in mice of the NB5092 strain, isolated from Nelson Bay, compared with the mouse virulent T48 strain isolated in north Queensland (Gard *et al.*,1973; Woodroofe *et al.*,1977). The extent of genetic divergence between these two strains is in the order of 2% as deduced from nucleotide sequencing studies of the entire genomes of RRV T48 and NB5092 (Faragher *et al.*,1988). There is no information relating genetic type of RRV to virulence in humans.

1.3.5 Pathogenesis of RRV infection

The pathogenesis of epidemic polyarthritis is not well understood. The failure to isolate virus from clinical cases and the presence of antibodies in patients at the time of clinical presentation initially led physicians to believe that the disease was immune-mediated with an incubation period of 9-12 days. However, the outbreak of epidemic polyarthritis in the Pacific demonstrated that the incubation period of the disease could be as short as 2-3 days, supporting a primary viral role in disease symptoms (Rosen *et al.*,1981). Activated macrophages and monocytes are prominent features of the inflamed joints (Clarris *et*

al.,1975; Fraser *et al.*,1981). In addition there is a genetic association of epidemic polyarthritis with HLA type (Fraser *et al.*,1980).

A limitation in experimental studies of RRV is the lack of an appropriate animal model; the commonly used mouse model for RRV pathogenesis does not produce polyarthritis. However, this animal has proved very useful for comparative studies on viral pathogenesis with the T48 and NB5092 strains of RRV (Murphy *et al.*,1973; Mims *et al.*,1973). T48 is rapidly lethal in mice up to three weeks old, killing virtually 100% of infected mice. NB5092 is relatively avirulent, killing less than 50% of neonatal mice infected with the virus and is asymptomatic in older mice. Outbred mice injected peripherally with RRV T48 or NB5092 developed extensive muscle necrosis and paralysis of the hind limbs. Lesions in the central nervous system (CNS) of NB5092 infected mice were minimal and did not coincide with clinical symptoms of hind leg paralysis. Neonatal mice infected with T48 died before CNS changes could be observed (Murphy *et al.*,1973; Mims *et al.*,1973). It was concluded that hind leg paralysis and death in mice was due to massive muscle damage. In contrast, Seay and Wolinski (1982) reported that seven-day old Balb/c mice injected peripherally with RRV T48 developed encephalomyelitis with focal necrosis in the cerebellum, brainstem and spinal cord in addition to severe myositis. From these studies it appears that strain and age of mice as well as strain of virus may be important in the pathogenesis of RRV in mice.

1.4 Alphavirus structure and genome organization

1.4.1 Alphavirus structure

The most extensive studies of alphavirus structure have been done with SIN and SFV which have very similar physical properties (reviewed by Harrison, 1986). Electron microscopy reveals a roughly spherical particle, approximately 60nm in diameter, with icosahedral symmetry.

The virion consists of a nucleocapsid core, containing the RNA genome, surrounded by a host derived, lipid membrane. Within the membrane are embedded glycoprotein spikes composed of the virus encoded proteins E1 and E2. A third glycoprotein, E3, is found in association with the glycoprotein spikes of SFV (Garoff *et al.*,1974). Small amounts of the viral 6K protein have been demonstrated in the membrane of SIN (Nitschko and Schlesinger, 1990).

Using cryoelectron microscopy of vitrified particles and computer generated reconstruction, Fuller (1987) determined the three dimensional structure of SIN at 3.5nm resolution. The spikes of the virion are columnar trimers of the E1 and E2 heterodimer, arranged on a T=4 lattice. The lipid bilayer is polyhedral and surrounds a smooth, fenestrated, T=3 nucleocapsid. Thus a complete SIN virion contains 240 copies of each of the spike proteins arranged as 80 trimeric spikes, and 180 copies of the capsid protein. This gives rise to two types of spike-capsid interactions. Spike trimers near the five-fold axes interact tightly with triplets of capsid elements whereas those on the three-fold axes interact more loosely (Fuller, 1987). Similar predictions have been made for SFV (Vogel *et al.*,1986). This is in contrast to the 1:1 capsid to spike heterodimer ratio previously predicted (von Bonsdorf and Harrison, 1975).

Details of the glycoprotein structure at high resolution are not known. The association of E1 and E2 as non-covalently linked heterodimers has been demonstrated for SFV (Ziemieki and Garoff, 1978) and SIN (Rice and Strauss, 1982). The studies with SIN demonstrated that three such heterodimers form a cross-linkable cluster in the viral surface thus linking the microscopy and chemical predictions (Harrison, 1986). E2 is more exposed on the virus surface as judged by susceptibility to iodination by lactoperoxidase (Sefton *et al.*,1973; Dalrymple *et al.*,1976). Chang and Trent (1987) have suggested that the less hydrophilic E1 may form the

inner core of the glycoprotein spike. In SIN, both E1 and E2 are glycosylated with each protein containing both simple and complex carbohydrate chains (Mayne *et al.*,1985). E1 of RRV, SFV, EEE and ONN contains a single glycosylation site (Garoff *et al.*,1980; Dalgarno *et al.*,1983; Chang and Trent, 1987; Levinson *et al.*,1990). The oligosaccharides are partly masked in the virus as measured by the ratio of glycosidase sensitivity of intact virus to sensitivity of detergent solubilized glycoprotein, suggesting that some of the glycosylation sites are on lateral surfaces of the projecting proteins, rather than at the outer tip (McCarthy and Harrison, 1977).

Hydrophobic sequences at the carboxy termini of E1 and E2 span the lipid bilayer, probably as an alpha helix (Garoff *et al.*,1980). Fuller (1987) has proposed that the columnar nature of the spikes in SIN suggests that the six transmembrane helices associated with the structure lie in a bundle beneath the stalk of the spike. This would stabilize the trimer and may be essential for its formation as Kielian and Helenius (1985) have shown that proteolytically derived ectodomains of the spike polypeptides are monomeric.

The lipid bilayer is host-derived and its composition reflects the cells in which the virus was grown (Laine *et al.*,1973; Luukonen *et al.*,1976; Luukonnen *et al.*,1977). Fuller (1987) has predicted that the bilayer is a polyhedral structure, bent at the position of the spikes, rather than a smooth sphere; this may require less distortion of the ratio of inner leaflet surface area to outer leaflet surface area during budding.

The nucleocapsid core consists of a complex of the capsid and viral RNA genome. The amino-terminal half of the capsid protein is poorly conserved among alphaviruses but is rich in arginine, lysine and proline residues, suggesting that this region interacts with the negatively charged

RNA. The remainder of the capsid protein is more strongly conserved and may interact with the cytoplasmic domain of E2 (Harrison, 1986). A specific binding site on the capsid protein for the cytoplasmic domain of E2 has been demonstrated using anti-idiotypic antibodies (Vaux *et al.*,1988). E1 has only a dipeptide on the cytoplasmic side of the membrane (Rice *et al.*,1982).

1.4.2 Structure of the alphavirus genome

The alphavirus genome consists of a single strand of positive polarity RNA with a sedimentation coefficient of 49S. This RNA is capped at the 5' end and polyadenylated at the 3' end (Strauss and Strauss, 1986). The complete genome has been sequenced for SIN, SFV, VEE, ONN and RRV (Strauss *et al.*,1984; Takkinen, 1986; Faragher *et al.*,1988; Kinney *et al.*,1989; Levinson *et al.*,1990); partial sequence data is available for MID, WEE and EEE (Strauss *et al.*, 1983; Hahn *et al.*,1988; Chang and Trent, 1987). The genome length is between 11-12 kb depending on the virus. The complete sequences of two strains of RRV (NB5092 and T48) have been determined (Faragher *et al.*,1988). NB5092 has a genome of 11,674 nucleotides while T48 is 11,883 nucleotides long. The difference in genome size results from insertions and deletions in the non-coding regions, but there are no differences in the size of the coding regions of the two strains. The genome organizations of RRV and other alphaviruses are similar. During alphavirus replication the infecting positive strand of 49S RNA serves as a template for the synthesis of a full length minus strand. This minus strand serves as a template for the synthesis of a 26S subgenomic RNA from which the structural proteins are translated and for genome length plus stranded RNA.

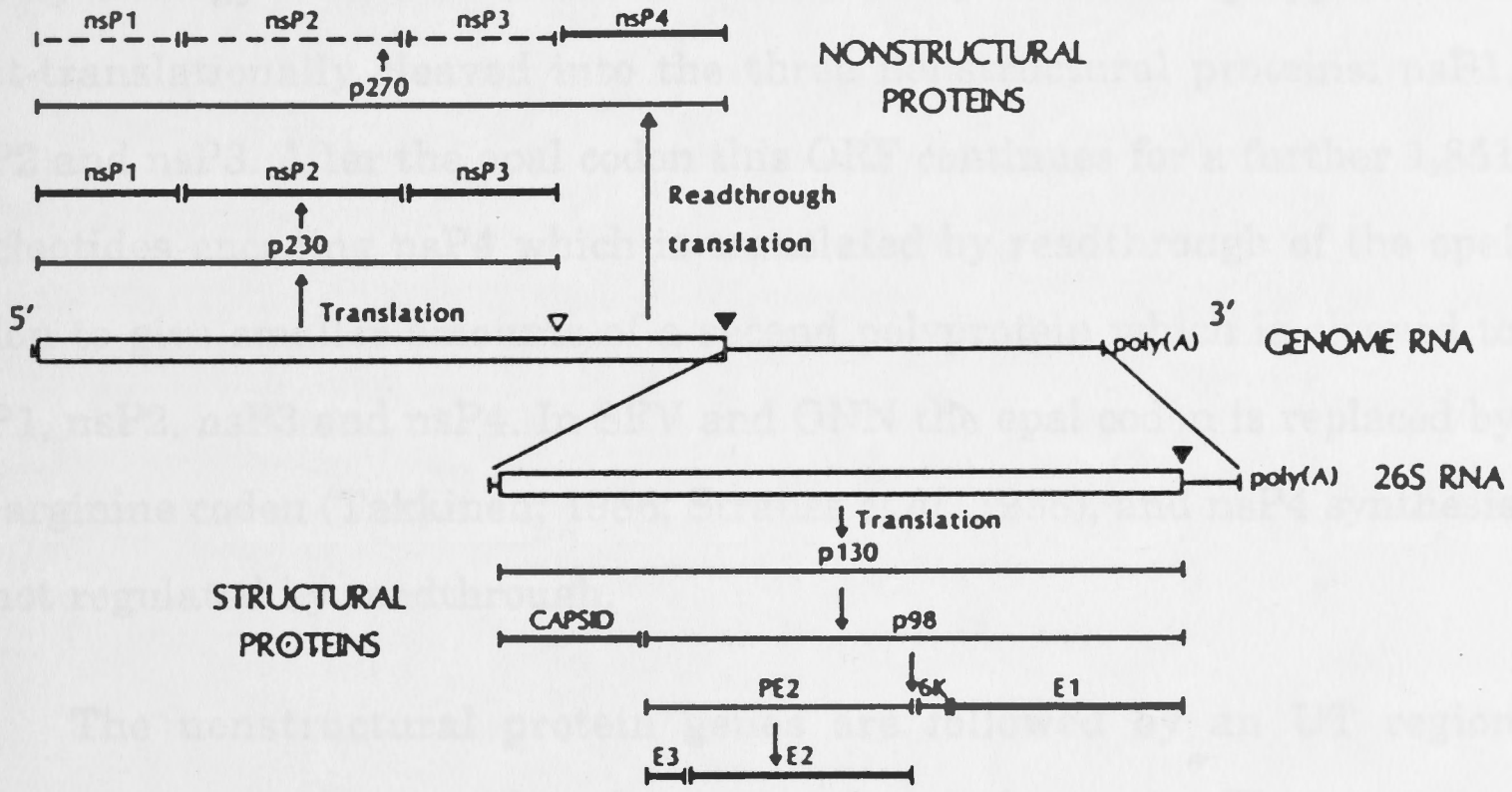
The SIN replication strategy is depicted in Fig 1.1. The 5' two-thirds of the genome encodes the nonstructural proteins which have a replicase

Figure 1.1

Alphavirus replication strategy*

A schematic representation of the translation and processing of the nonstructural and structural proteins of SIN. Translated regions of the genome and of subgenomic 26S RNA are shown as open boxes; untranslated regions are represented by single lines. Structural and nonstructural processing intermediates are indicated and the final protein products are shown as heavy lines. The nsP1, nsP2 and nsP3 produced by processing of the readthrough polyprotein are shown as dashed lines as their contribution to the pool of final products is probably minor. Triangles represent stop codons for termination of translation of the structural and nonstructural polyproteins; the opal codon between the nsP3 and nsP4 genes is represented by the open triangle.

* Adapted from Faragher (1987)


nsP1
nsP2
nsP3
nsP4
p270
NONSTRUCTURAL
PROTEINS
nsP1
nsP2
nsP3
p230
Readthrough
translation
Translation
5'
3'
poly(A)
GENOME RNA
poly(A)
26S RNA
Translation
p130
STRUCTURAL
PROTEINS
CAPSID
p98
PE2
6K
E1
E3
E2

function and must be translated first. The 3' third of the genome encodes the structural proteins and is translated from the subgenomic 26S RNA. For RRV, a 5' untranslated (UT) region of 80 nucleotides is followed by a single open reading frame (ORF) of 5,586 nucleotides terminated by an opal codon (UGA). This ORF encodes a polyprotein of 1,862 amino acids (Faragher *et al.*,1988). Little is known of the RRV nonstructural proteins but by analogy with SIN (Strauss and Strauss, 1986), the polyprotein is post-translationally cleaved into the three nonstructural proteins: nsP1, nsP2 and nsP3. After the opal codon this ORF continues for a further 1,851 nucleotides encoding nsP4 which is translated by readthrough of the opal codon to give smaller amounts of a second polyprotein which is cleaved to nsP1, nsP2, nsP3 and nsP4. In SFV and ONN the opal codon is replaced by an arginine codon (Takkinen, 1986; Strauss *et al.*,1988), and nsP4 synthesis is not regulated by readthrough.

The nonstructural protein genes are followed by an UT region between the nonstructural and structural protein genes. This contains three in-frame stop codons which ensure termination of the readthrough polyprotein. Differences in the position and number of stop codons in this junction region are found between RRV T48 and NB5092, SIN, SFV and MID (Faragher *et al.*,1988; Ou *et al.*,1982).

Translation of the structural polyprotein genes occurs from the 26S subgenomic RNA in a different reading frame to the nonstructural protein genes and begins 45 nucleotides, for RRV NB5092, or 48 nucleotides for RRV T48, from the 3' terminal nucleotide of the nsP4 gene (this nucleotide also represents the 5' terminal nucleotide of the 26S subgenomic RNA). For both NB5092 and T48 an ORF of 3,762 nucleotides encodes a structural polyprotein of 1,284 amino acids; this is followed by a 3' UT region of approximately 348 nucleotides for NB5092 and 524 nucleotides for T48 (Faragher *et al.*,1988).

1.4.3 Conserved sequences in alphavirus genomes

There is little conservation of nucleotide sequence between alphaviruses even though amino acid sequences may be reasonably conserved (Bell *et al.*,1984). Conserved nucleotide sequences must be under strong selection pressure during RNA replication and are likely to be involved as regulatory elements in replication and assembly (Strauss and Strauss, 1986). Four conserved regions of nucleotide sequence have been identified for alphaviruses. These are (1) a 19 nucleotide element adjacent to the poly-A tract at the 3' end of the genome; (2) a 51 nucleotide region in the 49S RNA approximately 150 nucleotides from the 5' end; (3) a 21 nucleotide sequence in the junction region between the nonstructural and structural protein genes and (4) a less conserved element at the 5' end of the genomic RNA (Strauss and Strauss, 1986).

The sequences at the 19 nucleotide region adjacent to the poly-A tail and the 5' 51 nucleotide element may be involved in minus strand synthesis by cyclization of the viral RNA and simultaneous recognition by the transcriptase complex (Ou *et al.*,1983; Strauss and Strauss, 1986; Levinson *et al.*,1990). Conservation of these sequences is important for efficient replication in both vertebrate and mosquito cells (Kuhn *et al.*,1990; Niesters and Strauss, 1990).

The 21 nucleotide element in the junction region includes the 19 nucleotides coding for the C-terminus of nsP4 and the first two nucleotides of the 26S RNA (Ou *et al.*,1982; Faragher *et al.*,1988; Levinson *et al.*,1990). It has been suggested that the complement of this sequence in the minus strand may be the promoter for transcription of 26S RNA (Ou *et al.*,1982).

The sequence at the 5' end of the genomic RNA is less conserved among alphaviruses than the previous three sequences (Strauss and

Strauss, 1986). The first 22 nucleotides are approximately 80% conserved between SIN and RRV (Faragher *et al.*,1988). The complement of this sequence is predicted to form a stem and loop structure in the 3' end of the genomic minus strand RNA which may be the replicase binding site for the initiation of plus strand RNA transcription (Strauss and Strauss, 1986).

Most alphaviruses have repeat sequences in the 3' UT region (Ou *et al.*,1981; Faragher and Dalgarno, 1986; Levinson *et al.*,1990). RRV NB5092 contains only a single copy of a 49 nucleotide repeat element found in four copies in T48 thus it is apparent that in a particular virus type, variations in the number of repeats can be accommodated. Site-directed mutagenesis has been used to examine the SIN 3' UT region (Kuhn *et al.*,1990). Almost all mutations in this region had a deleterious effect on virus replication and in many cases this effect was more severe in mosquito cells than avian cells. This suggests that although there is considerable sequence plasticity in the 3' UT region, the conservation of this region, and elements within it, is important for viral replication. The differential host effects suggest that host cell proteins may be involved in interactions with this region and that it may represent a compromise between sequences that allow the most efficient replication in mosquitoes and those that are most efficient in vertebrates.

The 51 nucleotide 5' sequence may be a recognition site for encapsidation as this would explain why 26S RNA is not encapsidated (Strauss and Strauss, 1986). This has been disputed by Weiss *et al.* (1989) who implicate a region between nucleotides 746-1,226 in SIN encapsidation. Mapping of defective interfering (DI) RNAs from SIN and SFV to define structures within the RNA required for RNA replication and encapsidation (reviewed by Schlesinger and Weiss, 1986) has shown that only sequences in the 162 nucleotide region at the 5' terminus (thus

excluding most of the conserved 5' 51 nucleotide region) and in the 19 nucleotide element at the 3' terminus are specifically required for replication and packaging of DI virus RNA (Levis *et al.*,1986).

1.5 Replication of alphaviruses

1.5.1 Cell attachment and entry

Alphavirus replication requires that the virus attach to and enter a susceptible cell. This is followed by penetration of the nucleocapsid into the cytoplasm, uncoating and translation of the RNA genome; RNA replication and translation are followed by virus assembly and release from the cell.

Like many other enveloped viruses, alphaviruses are believed to enter cells by receptor mediated endocytosis. SFV and, to a lesser extent, SIN, have served as the paradigms for most alphavirus entry studies (reviewed by Kielian and Helenius, 1986). Cultured cells are able to bind large numbers of SIN or SFV virions with estimates ranging from $5x10^4$ to $1.5x10^6$ particles bound per cell (Birdwell and Strauss, 1974; Fries and Helenius, 1979; Smith and Tignor, 1980). Pierce *et al.* (1974) demonstrated two types of binding using SIN; weak, in which virus could be washed from the cells with high ionic strength buffer and strong, which was virtually irreversible. It was suggested that weak binding involved ionic interactions between the virion and the cell surface while the strong binding involved receptor recruitment. Once tight binding occurs it is virtually irreversible (Marsh and Helenius, 1980). Based on experiments with proteases and neuraminidase, attachment sites on cultured cells are proteinaceous and sialic acid is not required for virus binding (Smith and Tignor, 1980).

No specific host-cell receptor for alphaviruses has been identified. Helenius *et al.* (1978) demonstrated binding of SFV glycoprotein spikes to human and murine histocompatibility antigens. However, Oldstone *et al.* (1980) showed that cells lacking these proteins could be productively infected with SFV and therefore that histocompatibility antigens were not required for alphavirus infection. This observation is supported by chemical cross-linking studies of SIN bound to cells which identified a 90kD protein as the major protein at or adjacent to the SIN binding site (Maasen and Terhorst, 1981). In all cases one must consider that the productive receptor may be different to the proteins identified in cell binding studies (Dimmock, 1982) and that the situation *in vivo* may be different again. In arthropod cells it has been argued (Brown and Condreay, 1986) that there is no evidence for a specific receptor-mediated entry. However, Hase *et al.* (1989), using electron microscopy, claim to have demonstrated receptor mediated endocytosis of SFV in mosquito cells.

In cultured cells, entry through the plasma membrane follows translocation of receptor bound virus to clathrin coated pits which are internalized as coated vesicles. Virus is delivered to endosomes where the acid pH triggers a conformational rearrangement of the glycoprotein spike leading to fusion of the endosomal and viral membranes which releases the nucleocapsid into the cytosol (Helenius *et al.*,1980; Marsh and Helenius, 1980; Kielian and Helenius, 1985). This fusion reaction is postulated to be analogous to that occurring with the influenza virus haemagglutinin during cell entry (Marsh and Helenius, 1989). Release of the nucleocapsid into the cytosol is concurrent with uncoating of the viral RNA which is translated to form the viral replicase/transcriptase.

1.5.2 Replication of the alphavirus genome.

The viral replicase complex must have at least four activities: (1) initiation of minus-strand synthesis, (2) initiation of genome length plus-strand synthesis, (3) initiation of subgenomic 26S RNA synthesis, and (4) elongation and completion of initiated chains (Strauss and Strauss, 1986). The four nonstructural proteins (nsP1, nsP2, nsP3 and nsP4) must function as the viral replicase/transcriptase complex, although an active replicase complex has not been isolated. Host cell components may also play a role in the viral replicase (Baric *et al.*,1983). By mapping temperature sensitive mutants of SIN which are defective in RNA synthesis at the non-permissive temperature (RNA$^-$ ts mutants), Hahn *et al.* (1989a) have shown that nsP4 probably contains the viral replicase activity. Initiation of minus strand synthesis maps to nsP1 (Hahn *et al.*,1989b), which has also been associated with SIN RNA methyltransferase activity involved in 5' capping (Mi *et al.*,1989). 26S RNA synthesis appears to be initiated by nsP2, which may also be involved in minus strand synthesis (Hahn *et al.*,1989b). The high net positive charge of this protein suggests that it may be involved in RNA binding (Levinson *et al.*,1990). In addition a protease activity required for cleavage of the nonstructural polyproteins appears to reside in nsP2 (Ding and Schlesinger, 1989; Hahn *et al.*,1989b). The functions of nsP3 are unresolved. The C-terminal region of this protein is highly variable between alphaviruses both in amino acid sequence and in length (Strauss *et al.*,1988; Davis *et al.*,1989; Kinney *et al.*,1989) and thus functional activity may reside in the N-terminal region. To explain the differences in regulation of nsP4 synthesis between SIN which has an opal stop codon at the 3' end of the nsP3 gene and SFV which has no opal codon and therefore synthesizes relatively large amounts of nsP4, it has been suggested that nsP34 may be the active form of nsP4 (Hardy and Strauss, 1988; Li and

Rice, 1989). Peranen *et al.* (1988) have demonstrated that nsP3 of SFV is a phosphoprotein and speculated that its phosphorylation may be involved in regulation of some RNA synthesis steps. Phosphorylation of this protein has also been demonstrated for SIN (Hardy and Strauss, 1988).

1.5.3 Translation of the 26S subgenomic RNA and processing of the structural proteins

Translation of the 26S subgenomic RNA is initiated at an AUG codon which is 45-48 nucleotides from the 5' end of the 26S RNA in RRV (Faragher *et al.*,1988). The capsid protein is translated first and cleaved from the nascent polypeptide chain by an autocatalytic activity believed to function as a serine protease (Hahn *et al.*,1985; Melancon and Garoff, 1987). This cleavage exposes the precursor to the E2 polypeptide (PE2, also known as P62) in which a hydrophobic leader sequence is recognized by the cellular signal recognition particle, forming a complex consisting of the nascent polypeptide, mRNA, ribosomes and signal recognition particle. This complex binds to receptors on the endoplasmic reticulum (ER), allowing transport of PE2 through the membrane (Bonatti *et al.*,1984). Glycosylation of PE2 occurs as the nascent polypeptide moves into the lumen of the vesicle (Schlesinger and Schlesinger, 1986). A hydrophobic sequence of, in RRV, 60 amino acids (the 6K protein) probably acts as a signal sequence to initiate transport of E1 across the membrane of the ER after proteolytic cleavage releases PE2 (Welch and Sefton, 1980). The 6K peptide is cleaved from E1, possibly by signal peptidase (Schlesinger and Schlesinger, 1986).

1.5.4 Host cell modification of virus proteins

Initial glycosylation of the E1 and PE2 proteins occurs in the rough endoplasmic reticulum (RER) (Sefton, 1977) by the attachment of mannose-rich side chains to asparagine residues located within potential

glycosylation sites consisting of Asn-X-Ser/Thr triplets. These oligosaccharides are processed by host enzymes in the ER and Golgi to form either complex oligosaccharides containing the peripheral sugars N-acetyl-D-glucosamine, galactose, sialic acid and fucose or a high mannose type (Kornfeld and Kornfeld, 1985). Intermediate types are also found (Davidson and Hunt, 1985) and the extent of processing depends on both the cell type and the position of the glycosylation site in the polypeptide (Keegstra *et al.*,1975; Burke and Keegstra, 1979; Hsieh *et al.*,1983a; Davidson and Hunt, 1983; Hsieh *et al.*,1983b). In mosquito cells complex oligosaccharides are not formed (Stollar *et al.*,1976; Luukonen *et al.*,1977; Hsieh and Robbins, 1984) indicating that the peripheral sugars in complex chains are not required for viral assembly or infectivity. The major role for added oligosaccharides may be in protein folding and stability (Schlesinger and Schlesinger, 1986).

Fatty acylation of E1 and PE2 occurs either late in the ER (Berger and Schmidt, 1985), in the cis or medial Golgi (Quinn *et al.*,1983) or in an intermediate compartment between the ER and the cis Golgi (Bonatti *et al.*,1989). Long chain fatty acids, mostly palmitic acid (Schmidt *et al.*,1979), are added to cysteine residues in the transmembrane domains of E1 and PE2. Non-fatty-acylated proteins are defective in virus assembly (Schlesinger and Malfer, 1982). The fatty-acyl groups may also have a role in membrane fusion (Schmidt and Lambrecht, 1985). In addition, the 6K protein is highly palmitoylated and this appears necessary for virus assembly and budding (Nitschko and Schlesinger, 1990; Nitschko *et al.*,1990).

1.5.5 Assembly, maturation and release of virus

The complexing of capsid proteins with the 49S RNA genome to form the nucleocapsid occurs rapidly and specifically; this is possibly connected

with the demonstrated binding of the capsid protein to the 60S ribosomal subunit, which process may have a role in virus assembly (Ulmanen *et al.*,1976). The capsid protein does not bind to the 26S viral RNA (Ulmanen *et al.*,1976). Coombs and Brown (1989) have suggested that the SIN capsid protein packages segments of the genome into nucleoprotein beads to form the nucleocapsid structure.

During transport through the cellular organelles to the plasma membrane, PE2 is cleaved to E2 and E3 by a trypsin-like cleavage adjacent to a pair of basic residues. The timing of this cleavage and the enzyme involved are not clearly defined. While a cathepsin type protease in the Golgi has been proposed (Dalgarno *et al.*,1983; Schlesinger and Schlesinger, 1986), Naim and Koblet (1988) and Knipfer and Brown (1989) have raised the possibility that some cleavage of PE2 occurs earlier in the RER. This cleavage may be due to a host enzyme which is not restricted to a particular compartment or to virus mediated proteolysis. In contrast, cleavage of PE2 late in the trans Golgi or in a post-Golgi compartment is suggested by the results of de Curtis and Simons (1988). It appears that this event may occur in a number of compartments or in a continuous manner depending on the system examined.

Cleavage of PE2 results in structural alterations to the heterodimer and may be essential for maturation of alphaviruses (Schlesinger and Schlesinger, 1986). However, a SIN mutant with a glycosylation site introduced at the PE2 cleavage point produced infectious virus with similar titres to control SIN even though the PE2 cleavage was abolished (Russell *et al.*,1989). Presley and Brown (1989) used low doses of the ionophore monensin to inhibit cleavage of PE2 and demonstrated PE2 incorporation into infectious virus, replacing E2. Lobigs and Garoff (1990) showed that cleavage of PE2 is essential for the exposure of the fusion function of the SFV viral spike, suggesting that cleavage of PE2 exerts a

conformational effect on E1 making it susceptible to low pH. Following PE2 cleavage, E3 remains non-covalently associated with the E1/E2 heterodimer of SFV but not of SIN or RRV (Mayne *et al.*,1984; Martin *et al.*,1979).

Viral glycoproteins are thought to arrive at the plasma membrane via transport vesicles which fuse with the cell surface (Garoff *et al.*,1982). E1 was not transported to the cell surface when an aberrant form of PE2 was produced by a ts mutant of SFV at the non-permissive temperature, but was retained in the microsomal vesicles (Hashimoto *et al.*,1981). On this basis it has been suggested that PE2 is required for transport of E1 to the plasma membrane (Schlesinger and Schlesinger, 1986). A model for virus assembly and budding proposes an initial interaction between the nucleocapsid and a few glycoprotein spikes at the cell surface to form a "nucleation patch". Additional glycoprotein spikes would then translocate into this patch with concomitant displacement of host membrane proteins. Protein-protein interactions between additional molecules of E2 and the capsid proteins would drag the plasma membrane around the spherical nucleocapsid, leading to fusion of the bilayers and release of virions (Garoff and Simons, 1974). A similar model has been proposed by Vogel *et al.* (1986). In support of this model an E2-capsid protein association has been demonstrated for SFV with cross-linking reagents (Ziemiecki and Garoff, 1978) and using monoclonal anti-idiotypic antibodies (Vaux *et al.*,1988). Alterations in monovalent cation transport associated with the appearance of virus glycoproteins in the membrane may facilitate virus release (Ulug *et al.*,1989). This is consistent with the suggestion that the charge of the viral envelope is a major determinant in virus release (Strauss *et al.*,1980; Garry *et al.*,1985).

1.6 Functional domains of alphavirus glycoproteins E1 and E2 involved in cell entry

The alphavirus glycoproteins E1 and E2 form the external surface of the virus and must be involved in such functions as cell attachment, recognition of cell surface receptors and membrane fusion. Their role in alphavirus assembly has already been discussed. Because these proteins mediate receptor recognition they could also be virulence determinants at least partly defining cell and tissue tropism and vector specificity. As E1 and E2 exist as a heterodimer (Ziemieki and Garoff, 1978), the structure of which may be further modified by the quaternary conformation of the viral spike, it is likely that the functional dissection of E1 and E2 may lead to oversimplification when determinants on both proteins are required for certain functions.

The structural protein genes for a number of alphaviruses have been sequenced (SIN, SFV, RRV, VEE, WEE, EEE and ONN; Rice and Strauss, 1981; Garoff *et al.*,1980; Dalgarno *et al.*,1983; Johnson *et al.*,1986; Hahn *et al.*,1988; Chang and Trent, 1987; Levinson *et al.*,1990) and the apparent organization in terms of ectodomains, membrane spanning domains and cytoplasmic domains is conserved. Bell *et al.* (1984) analysed the N-terminal amino acid sequence of the glycoproteins of eight alphaviruses and demonstrated that although the sequence varied between viruses, residues important in secondary structure such as cysteines, prolines, glycines and aromatic amino acids tended to be conserved suggesting that the three dimensional structures of E1 and E2 are similar in all alphaviruses. Examination of the complete, colisted, amino acid sequences for E1 and E2 of RRV, SFV and SIN (Dalgarno *et al.*,1983) supports this conclusion. As the three dimensional structure of the alphavirus spike glycoproteins has not been determined, the definition of functional

domains on E1 and E2 is currently limited to the linear amino acid sequence.

The respective roles of E1 and E2 in receptor binding have not been resolved. As the two proteins exist as a heterodimer in a trimeric spike, interactions with receptors may involve sites on both proteins and/or complex conformational rearrangements. Removal of E2 from SFV virions by protease digestion produced particles containing predominantly E1 but which had virtually normal infectivity. This suggests that E1 alone is sufficient for infection of cultured cells (Omar and Koblet, 1988). However, it is unclear whether this is receptor mediated or results from exposing a fusion domain on E1 that interacts directly with the cell plasma membrane. *A priori* it would seem that the potential for exposing cryptic domains on E1 following the removal of E2 is high. Antiserum to E2 but not to E1 inhibits binding of VEE to cells. In addition some E2 specific monoclonal antibodies (mAbs) inhibit virus attachment to cells in tissue culture (Roehrig *et al.*,1988). Further evidence of a role for E2 in cell entry is provided by SIN variants selected for rapid penetration of BHK cells. The increased rate of penetration of most of these variants is caused by an amino acid alteration at amino acid 114 of E2 (Ser→Arg), suggesting that this protein is able to modulate cell entry (Davis *et al.*,1986; Polo *et al.*,1988; Russell *et al.*,1989). Rapid penetrating variants of RRV T48 with changes at amino acids 246 and 248 of E2, have also been described (Vrati, 1986).

There is good indirect evidence that E1 is the viral fusogen. This has been demonstrated with fusion mutants of SIN (Boggs *et al.*,1989), in SFV with E2 proteolytically removed (Omar and Koblet, 1988) and in recombinant expression systems (Kondor-Koch *et al.*,1983). E1 is also responsible for haemolysis of erythrocytes *in vitro* (Yamamoto *et al.*,1981; Lenard and Miller, 1981; Väänanen and Kääriänen, 1979). This appears to be analogous to alphavirus induced cell fusion (Väänanen and Kääriänen,

1980). Haemagglutinating activity is associated with E1 for alphaviruses (Dalrymple *et al.*,1976; Helenius *et al.*,1976; Simizu *et al.*,1984), however, E2 is the viral haemagglutinin for VEE (France *et al.*,1979). These functions of E1 all relate to fusion with cell membranes and do not require cellular proteins (Mooney *et al.*,1975) although there is an absolute requirement for cholesterol or other β-OH sterols and mildly acid pH (Mooney *et al.*,1975; White and Helenius, 1980; Kielian and Helenius, 1984).

It has been postulated (Garoff *et al.*,1980; Rice and Strauss, 1981) that fusion activity resides in a hydrophobic domain near the N-terminus of E1. In RRV this consists of the 17 amino acids forming residues 80-96, which are identical to the corresponding region in SFV and only differ from SIN in three amino acids (Dalgarno *et al.*,1983). This domain is in a region of 58 amino acids which is almost entirely conserved between RRV and SFV, with only one (conservative) amino acid substitution. This degree of conservation is far greater than the overall level (75%) between these two viruses (Dalgarno *et al.*,1983). Chang and Trent (1987) have postulated that for EEE, a seven amino acid sequence located at the C-terminus of this region and in a predicted antigenic domain, may interact with the cellular receptor and induce changes in the fusion domain leading to penetration. Omar and Koblet (1988) have predicted that there is more than one hydrophobic peptide mediating fusion. This is supported by the observation that haemolytic activity can be separated from haemagglutinating activity on E1 using mAbs (Chanas *et al.*,1982). Monoclonal antibodies to E2 can also block haemagglutination and this may be a function of the E1/E2 heterodimer in intact virus (Schmaljohn *et al.*,1983; Boere *et al*,1984).

Currently a case can be made for either E1 or E2 being involved in receptor recognition, however, it seems likely that the fusion component of cell entry involves E1.

1.7 Definition of neutralizing antigenic determinants on alphavirus glycoproteins

Neutralization determinants (epitopes) on viral proteins are a subset of antigenic sites which are functionally important because antibodies recognizing these sites provide protection from viral infection and may be involved in recovery from infection (Casals, 1963). Thus the identification of neutralization epitopes and an understanding of the mechanism of neutralization are important for the design of vaccines. Neutralization epitopes may also define functional domains involved in virus replication. For example sites on E2 involved in penetration in tissue culture of SIN and RRV show alterations in neutralizing mAb reactivity (Olmsted *et al.*,1984; Olmsted *et al.*,1986; Russell *et al.*,1989; Vrati, 1986).

Polyclonal antisera raised against intact or partially denatured virus reflect the specificities and affinities of the total antibody population and are thus not useful for examining or defining single regions of viral proteins or specific determinants such as neutralization epitopes. Antisera raised against purified E1 or E2 of SIN showed that neutralizing antibodies were directed to E2 while E1 had haemagglutinating activity (Dalrymple *et al.*,1976). In contrast the use of mAbs has enabled definition and study of individual epitopes and domains of E1 and E2 (reviewed by Roehrig, 1986). The selection of mAb resistant variants (escape mutants) has allowed the mapping of these epitopes on the linear amino acid sequence of the structural proteins.

For RRV, three neutralization epitopes have been mapped on E2 by selecting mAb escape mutants and competition binding assays (CBAs) (Vrati *et al.*,1988). Five mAbs were used in CBAs to define three overlapping epitopes; a (defined by mAbs T10C9 and T4D2), b (mAbs NB3C4 and T1E7) and c (mAb T1D11). Epitopes a and c were topographically

distinct but were united by epitope b. Three of these mAbs (T10C9, NB3C4 and T1E7) were used to select neutralization resistant variants of both RRV T48 and RRV NB5092. Nucleotide sequencing defined amino acid changes on E2 which abolished neutralization by the selecting mAb. These were: E2 216 for mAb T10C9 (epitope a); E2 232 and 234 for mAb NB3C4 (epitope b1); and E2 246, 248 and 251 for mAb T1E7 (epitope b2). These epitopes are within 30 amino acids of each other in the primary sequence of E2 and are located between the two predicted glycosylation sites at Asn 200 and Asn 262 in hydrophilic regions of the protein. Other sites can also influence neutralization; for example mAb NB3C4 is affected by a deletion of seven amino acids between residues 55-61 of E2 (Vrati *et al.*,1986).

Four neutralization determinants have been identified on SIN, one on E1, (Chanas *et al.*,1982; Schmaljohn *et al.*,1983; Stec *et al.*,1986) and three on E2 (Roehrig *et al.*,1982; Schmaljohn *et al.*,1983; Stanley *et al.*,1985; Stec *et al.*,1986; Olmsted *et al.*,1986; Davis *et al.*,1987). The E2 sites have been mapped to residues 181, 186, 190 (epitope E2a), 214 and 216, (epitope E2b) and 62, 96 and 159, (epitope E2c) by selection of escape mutants (Strauss *et al.*,1987; Stec *et al.*,1986; Davis *et al.*,1987; Pence *et al.*,1990). Like RRV, sites outside these defined regions can affect neutralization of SIN; for example a Ser114→Arg change in E2 affects epitope E2c (Davis *et al.*,1986).

Antigenic sites of SFV, VEE and WEE have been mapped topographically using CBAs (Boere *et al.*,1984; Roehrig and Mathews, 1985; Hunt and Roehrig, 1985) but no sequence data on escape mutants has been published.

The mechanism of virus neutralization by antibodies is poorly understood (for reviews see Dimmock, 1984, 1987; McCullough, 1986). Neutralization may depend not only on properties of the virus but also the

antibody and the host cell; mechanisms established *in vitro* using cultured cells may have little relationship to those occurring *in vivo* where other aspects of the immune system are involved (Schmaljohn *et al.*,1982; Dimmock, 1984).

A general mechanism of alphavirus neutralization by polyclonal antisera, based on *in vitro* experiments with neutralization of VEE by mAbs, has been proposed (Roehrig *et al.*,1988). This model envisages that in an immune host, antibodies of varying specificities act to limit virus dissemination. Some neutralizing antibodies bind to E2 at epitopes involved in cell attachment and block virus association with cell receptors. Virions not neutralized in this way may be neutralized by antibodies of other specificities for which the mechanism of neutralization is not obvious. For example, binding of some mAbs can alter the virus glycoprotein conformation such that the virus-receptor interaction is enhanced and stabilized on some cell types, even though the virus is effectively neutralized. Virus infectivity is efficiently neutralized, possibly analogously to post-attachment neutralization in flaviviruses (Gollins and Porterfield, 1986). Virions that escape neutralization prior to attachment may be neutralized by binding of antibody post-adsorption. Those cells supporting a productive virus infection can be destroyed, probably via immune cell mechanisms, following non neutralizing antibody binding to virion epitopes expressed on the infected cell surface (Schmaljohn *et al.*,1982).

1.8 The role of alphavirus glycoproteins in virulence determination

Virulence of a virus may be defined by the severity of disease caused in a particular host. This will reflect a combination of host factors including age, nutritional status, concurrent infections and genetic type and virus factors such as speed of replication, cell and tissue tropism.

Studies of alphavirus virulence typically use the mouse as a laboratory model and frequently use virus strains that are mouse adapted. The disease produced in mice may or may not relate to that seen in humans or other animals in the field and thus laboratory studies of alphavirus pathogenesis must be interpreted cautiously if used as a model for clinical disease.

Studies with RRV (Faragher *et al.*,1988) and VEE (Kinney *et al.*,1989) have compared the complete genome sequences of virulent and attenuated strains of these viruses. For RRV there were 284 nucleotide differences representing 48 amino acid differences between the mouse virulent T48 strain and NB5092 which is of low virulence for mice. In addition there were deletions and insertions in the non-coding regions. Such a large number of alterations make determination of virulence factors very difficult. In RRV T48 a deletion of amino acids 55-61 of E2 is associated with reduced virulence in mice (Vrati *et al.*,1986) supporting the concept that E2 is a determinant of virulence. There were only 11 nucleotide changes between the attenuated TC-83 strain of VEE, derived by passage in tissue culture, and its virulent parent. Six of these were coding changes, leading to five nonconservative amino acid changes in E2 and one conservative amino acid change in E1. This strongly points to E2 as a primary determinant of virulence in VEE. However, a single nucleotide alteration in the 26S RNA 5' untranslated region may to be involved in reduced RNA synthesis and decreased early virus production *in vivo* suggesting that this may be the mechanism of attenuation (Mecham and Trent, 1983; Johnson *et al.*,1986).

The low mouse virulent strain of RRV, NB5092 can be rapidly selected for virulence by serial passage in mice (Taylor and Marshall, 1975a; Meek *et al.*,1989). Variants selected in this way had amino acid changes in E2. These changes were not essential for increased virulence

as plaques picked from passage of virus without the changes in E2 were virulent. Virulence enhancement probably proceeded via an alteration in the nonstructural proteins, leading to increased replicative efficiency in mice, followed by selection for changes in E2. These changes in E2 may further enhance virulence, possibly by altering tissue tropism (Meek *et al.*,1989).

The virulence in mice of a number of strains of SIN has been characterized (reviewed by Griffin, 1989). Virulence depends on the strain of virus, age of mice and route of inoculation; most strains are lethal for neonatal mice by either the subcutaneous or intracerebral route of inoculation; all strains of SIN are avirulent in adult mice inoculated subcutaneously, however, some strains are lethal for adult and weanling mice inoculated intracerebrally. The neurovirulent NSV strain, selected by passage in mouse brain, is lethal for weanling mice by intracerebral inoculation. This increased virulence appears to be due to enhancement of viral replication in neurons rather than to a fundamental change in tropism (Griffin, 1989). The parental and the neurovirulent strains can be distinguished by several mAbs (Stanley *et al.*,1985). Nucleotide sequencing showed two amino acid changes in each of E1 and E2 (Lustig *et al.*,1988). In a complex series of experiments, the use of a transcribable complete cDNA clone of the SIN genome (Rice *et al.*,1987) allowed changes in the NSV structural glycoproteins to be made against a constant genetic background (Lustig *et al.*,1988). This showed that the changes in both E1 and E2 of the NSV strain were required for neurovirulence in weanling mice but regions of the genome outside the envelope glycoproteins were also involved in determining virulence (Lustig *et al.*,1988).

Selection of SIN (Baric *et al.*,1981; Olmsted *et al.*,1984; Russell *et al* 1989) and VEE mutants (Johnston and Smith,1988) with accelerated penetration in tissue culture coselects for reduced virulence in mice. In

SIN this is associated with a change in mAb neutralization involving the E2c epitope (Olmsted *et al.*, 1984; Olmsted *et al.*,1986; Russell *et al.*,1989) due to an E2 Ser114→Arg change (Davis *et al.*,1986). This is the only change necessary for both attenuation and increased penetration (Polo *et al.*,1988). Russell *et al.* (1989), using another strain of SIN showed that some fast penetrating variants failed to cleave PE2 to E2 and E3 due to the introduction of a glycosylation site at the cleavage point. This was associated with a loss of neurovirulence for both neonatal and adult mice mice. Mutants of this strain with the Ser114→Arg change were also selected, these variants were attenuated in neonatal mice by subcutaneous inoculation but remained virulent in both ages of mice by intracerebral injection (Russell *et al.*,1989).

Thus a number of factors including the envelope glycoproteins may determine alphavirus virulence. Changes in mAb reactivity are often correlated with changes in virulence indicating that alterations in surface proteins have occurred. Similar complexity with regard to virulence exist for other viruses. For poliovirus loci involved in attenuation are distributed in several regions of the genome including the 5' UT region, and altered antigenicity correlates poorly with virulence changes (Evans *et al.*,1985; Omata *et al.*,1986; Kawamura *et al.*,1989; Westrop *et al.*,1989; Moss *et al.*,1989). In contrast mAb resistant variants of rabies virus with a single amino acid change from arginine or lysine at position 333 in the surface glycoprotein are attenuated for mice (Tuffereau *et al.*,1989). The practical importance of identifying changes that are involved in attenuation lies in the potential instability of live attenuated vaccines if only one or two mutations are required for reversion to virulence. Changes involved with altered virulence may also provide insight into the mechanisms of disease pathogenesis and evolution of new viruses and diseases.

1.9 Evolution and adaptation of alphaviruses

1.9.1 Evolution of RNA viruses

In contrast to the highly stable DNA genomes of eukaryotes, where the rate of nucleotide substitutions, in functional genes, averages only about 10^{-9} nucleotide substitutions per site per year (Holland *et al.*,1982), RNA viruses have a very high rate of mutation. This has been estimated for a number of RNA viruses (reviewed by Smith and Inglis, 1987) and depending on the virus and the method used, a base misincorporation frequency of around 10^{-3}-10^{-5} per genome replication occurs. For alphaviruses estimates range from 10^{-3} to $<10^{-6.8}$ (Stec *et al.*,1986; Durbin and Stollar, 1986). The high error rate is probably due to a lack of proof-reading by the RNA polymerases involved in viral replication (Holland *et al.*,1982) although such an activity is claimed for the influenza virus RNA polymerase (Ishihama *et al.*,1986).

Based on studies with the bacteriophage Qβ, Domingo *et al.* (1978) have proposed that an RNA virus population is in a dynamic equilibrium with viable mutants arising at a high rate but being strongly selected against in competition with the wild-type. This has been termed a "quasispecies" in which the genome is statistically defined but individually indeterminate, in that most individual genomes will differ at one or more positions from the population consensus sequence. Such heterogenous populations should be capable of rapid adaptation to a changing environment because of the high proportion of variants in the population (Domingo *et al.*, 1978). Given the very high potential evolution rate of RNA viruses it is obvious that some constraints must act on the population. The need for conservation of protein structure and function, glycosylation sites, codon preferences, RNA secondary structure and conserved sequences involved in protein recognition act as powerful selective forces to stabilize

virus populations (Strauss and Strauss, 1986; Steinhauer and Holland, 1987).

1.9.2 Evolution and divergence of alphaviruses

It has been suggested, on the basis of genome organization, sequence similarities in the replicase proteins and the folding of the capsid proteins, that the positive strand RNA viruses have evolved from a common ancestor as a result of recombination, mutation and selection (Strauss and Strauss, 1988). Thus a number of apparently unrelated plant viruses have been postulated to be members of a "sindbis like" superfamily. In addition SIN has been likened to an enveloped picornavirus on the basis of similarities between its capsid protein sequence and predicted folding and those of the VP3 capsid proteins of picornaviruses (Fuller and Argos, 1987).

Nucleotide and amino acid sequence comparisons of alphaviruses reveal that even in regions where amino acid sequence is conserved, the codon usage has been essentially randomized (Strauss and Strauss, 1986). Thus the evolutionary divergence among alphaviruses is extremely extensive and the conservation of amino acid sequences presumably results from functional requirements (Bell *et al.*,1984).

By comparing the amino acid sequences available for the structural and nonstructural proteins for SIN, SFV, RRV, ONN, VEE, WEE, EEE and MID, Levinson *et al.* (1990) have proposed phylogenetic trees for each protein. When the E2 sequences are compared, the viruses form similar subgroups to those predicted by serology (Calisher *et al.*,1980; see Table 1.1). This is expected as serological comparisons based on differences in neutralizing epitopes will predominantly reflect differences in E2. When E1 and the capsid protein are considered the subgroups are less distinct; SFV, RRV and ONN form the SFV subgroup; EEE and VEE belong to the

EEE subgroup; SIN defines a separate subgroup and, based on limited data, MID forms a unique subgroup. WEE is in a special category as it is a recombinant virus (Hahn *et al.*,1988; see below).

Studies with RRV have shown the existence of a range of genetic and antigenic types (Faragher *et al.*,1985; Woodroofe *et al.*,1977). Similar studies have been performed for SIN (Olsen and Trent, 1985), the VEE complex (Young and Johnson, 1969; Trent *et al.*,1979), Getah (Morita and Igarishi, 1984) and the WEE complex (Trent and Grant, 1980) and have also demonstrated a range of genetic types. Alphaviruses appear to evolve by divergence in isolated ecological niches over substantial, but unknown, time-frames with one estimate of nucleotide divergence being a maximum of 0.1-0.2% per year (Hahn *et al.*,1988). Members of the subgroups defined by Levinson *et al.*,(1990) seem to be evolving at the same rate, however, individual proteins appear to evolve at different rates; nonstructural proteins diverge more slowly than structural proteins, while E2 evolves more rapidly than E1 and the capsid protein (Levinson *et al.*,1990).

Whether this divergence is simply by genetic drift that is, the gradual accumulation of random mutations or by adaptation and selection in new hosts and vectors probably depends on the circumstances. In practice a combination of both possibly operates. Given that transmission between a vertebrate host and a mosquito vector often involves very low numbers of virions (Burness *et al.*,1988) the potential of the "founder effect" (reviewed by Smith and Inglis, 1987) may be quite high in alphavirus evolution.

The requirements for replication in a mosquito vector and a vertebrate host may exercise a powerful constraining force on alphavirus evolution. To become fixed in the population, any mutation which enhances replication in one host must not be selected against in the second

(Burness *et al.*,1988). Kuhn *et al.* (1990) have demonstrated that changes in the 3' UT region of SIN can have a differential effect on virus replication in mosquito cells and vertebrate cells suggesting that regulatory sequences may reflect a compromise between the most efficient nucleotide sequence in host and vector. With RRV, Taylor and Marshall (1975b) demonstrated that alternating passages of the low virulence NB5092 strain between mice and mosquitoes did not select for changes in mouse virulence whereas passage in mice alone rapidly selected for virulence. In support of this, analysis of the E2 gene of RRV from a number of isolates made during a major epidemic in the Pacific islands demonstrated that only a single nucleotide change, altering amino acid 219, occurred in the course of the epidemic (Burness *et al.*,1988). Viruses which do not alternate between different hosts, on the other hand, may undergo high rates of evolution during epidemics (Kew *et al.*,1981; Nottay *et al.*,1981; Takeda *et al.*,1984; Evans *et al.*,1985; Buonagurio *et al.*,1986; Hahn *et al.*,1986).

Recently Hahn *et al.* (1988) have convincingly demonstrated that WEE is a recombinant between an EEE-like virus and a SIN-like virus. This is the first evidence of recombination between alphaviruses. It explains the paradoxical serological and pathological properties of WEE and indicates the importance of the EEE nonstructural and capsid components of the virus in causing encephalitis compared to the SIN-like glycoproteins. Recombination, rather than gradual evolutionary divergence, is a potentially powerful mechanism for the emergence of new types of alphaviruses (Levinson *et al.*,1990).

1.9.3 Adaptation and selection of alphaviruses in tissue culture

Passaging in tissue culture is a common method of preparing attenuated or laboratory adapted strains of viruses. Examples of successful vaccines produced in this way include the yellow fever 17D strain (Theiler

and Smith, 1937), the Sabin oral poliovirus vaccine strains (Sabin, 1965) and, for alphaviruses, the TC-83 vaccine strain of VEE (Berge *et al.*,1961). Passaging in tissue culture appears to select variants, either pre-existing in the virus population or arising by mutation, which eventually outgrow the wild-type to become the dominant population. This may produce a population which is altered in its growth rate and tissue tropism and may thus be attenuated for the natural host.

The molecular basis of selection in tissue culture is unclear and probably varies between viruses. To grow in a particular cell line a virion must be able to attach to the cell surface, bind to a productive receptor, enter the cell, replicate, assemble and escape from the cell. Adaptation to growth in a particular cell line may select variants with enhanced recognition of a cell surface receptor or with the ability to overcome some other barrier to cell recognition and entry. For alphaviruses this may be evident as changes in the surface glycoproteins E1 and E2. Some possible examples of this are given below.

Adaptation of SIN to mouse plasmacytoma cells was associated with an increased negative surface charge on the virion leading to an enhanced ability to initiate infection of these cells (Symington and Schlesinger, 1975; Symington and Schlesinger, 1978). Changes occurred in both E1 and E2 based on biochemical analysis, but no sequence data was given. Similarly enhanced infectivity was obtained by incubating the cells with heparin suggesting that surface charge on cells and virions was involved in cell attachment and entry (Symington and Schlesinger, 1978). In a second example, the attenuated TC-83 strain of VEE was generated by passaging the virulent TRD strain in tissue culture; after 45 serial passages in guinea pig heart cells the TRD strain had become avirulent for mice (Berge *et al.*,1961). There were 11 nucleotide differences between the TC-83 and TRD genomes, six of which resulted in amino acid changes Of these, five

occurred in the E2 protein and all five were non-conservative (Kinney *et al.*,1989). Finally, selection of SIN for rapid release or rapid penetration in BHK cells was associated with changes at either amino acid 114 of E2 or the introduction of a glycosylation site at amino acid one of E2 which prevented cleavage of PE2 (Davis *et al.*,1986; Russell *et al.*,1989).

E2 is the most variable alphavirus protein and appears to be largely responsible for strain evolution (Levinson *et al.*,1990). It is possible that E2 is important in adaptation to new hosts and that changes in this protein reflect this. Definition of such changes could help to define functional domains on E2.

1.10 Scope and outline of the thesis

Alphaviruses, particularly SIN and SFV, provide a well characterized system for study of the interactions of arboviruses and cells at the molecular level. In addition these viruses are frequently important pathogens of humans and other animals (Shope *et al.*,1980) and have been used in extensive studies of viral pathogenesis. There are, however, many unanswered questions about alphavirus biology and molecular virology. The natural cycling between vertebrate and invertebrate hosts raises many fascinating issues particularly with regard to cell receptors and protein structure and function. Answers to these may illuminate the mechanisms involved in alphavirus evolution, adaptation to new ecological conditions, the maintenance of enzootic foci and development of epidemics.

RRV is the most important alphavirus in Australia and surrounding regions causing frequent disease outbreaks (Marshall and Miles, 1984). In recent years it has been well characterized genetically (Dalgarno *et al.*,1983; Faragher *et al.*,1985; Faragher and Dalgarno, 1986; Faragher *et al.*,1988) and antigenically (Vrati *et al.*,1988). This has provided the opportunity for further study at the level of molecular and cell

biology. This thesis describes studies of the E2 glycoprotein of RRV; its role in immunogenicity, cell entry and its response to serial passage in cell culture.

In Chapter 2 studies on the antigenic structure of RRV are described. Neutralization of mAb resistant variants of RRV, altered in one or three neutralizing epitopes, by polyclonal antisera raised against the parental virus was examined to test the hypothesis that these epitopes represented an immunodominant site on RRV (Vrati *et al.*,1988). In further studies four short synthetic peptides were used to examine the antigenic structure of this neutralization domain on RRV. This was done by raising antisera to the peptides in mice and examining the reactivity of these antisera with RRV in a variety of immunological assays. Peptides were also examined for their ability to bind to antibodies raised to RRV.

The study of this neutralization domain is continued in Chapter 3 by measuring the rate of penetration in tissue culture of variants of RRV resistant to individual mAbs and thus with single amino acid alterations in E2. The penetration rate of variants with changes in epitopes a, b1 and b2 were determined in Vero and BHK cells and compared with the rates for the parental T48 and several field isolates of RRV. Growth kinetics and RNA synthesis kinetics in tissue culture were also examined. Binding studies with RRV were performed on cultured cells. In addition further variants of RRV resistant to mAb T1E7 were selected in an attempt to map permissible amino acid changes at this epitope.

Chapter 4 continues the examination of the envelope glycoproteins by determining the changes that occurred in these proteins on passaging RRV in chick embryo fibroblasts. RRV replicates poorly in birds (see earlier) and it was reasoned that by passaging the virus in avian cells insights into structure-function relationships of the glycoproteins might be

developed. It was of particular interest to determine whether changes occurred in the neutralization epitopes already defined on RRV E2 and implicated in penetration. The passaging of RRV in avian cells is described together with the results of antigenic, biological and genetic analysis of passaged variants.

In Chapter 5 a similar approach is taken by passaging RRV strains in human and arthropod cell lines. Passaged virus populations were subjected to genetic and antigenic analysis of the E2 gene and changes in this gene compared with those found following passage in avian cells.

Chapter 6 provides a general discussion of the work presented in this thesis.

Chapter 2

Studies on a neutralization domain of RRV E2 using anti-viral and anti-peptide antibodies

2.1 Introduction

Virus neutralizing antibodies are regarded as being of primary importance in recovery and protection from alphavirus infections (Casals, 1963). During such infections antibodies are produced to both E1 and E2 but neutralizing antigenic determinants are found predominantly on E2 (Dalrymple *et al.*,1976). However, some neutralizing mAbs are directed to sites on E1 (Chanas *et al.*,1982; Schmaljohn *et al.*,1983; Boere *et al.*,1984). The process by which antibodies neutralize alphaviruses is poorly understood (see Chapter 1), and characterization of the structure and function of neutralization determinants may help in understanding the mechanism of neutralization and the functional importance of neutralizing epitopes.

Three neutralization determinants have been defined on E2 of RRV T48 and NB5092 by the selection of variants resistant to neutralizing mAbs (Vrati *et al.*,1988). These epitopes, designated a, b1 and b2, are defined by single amino acid changes in E2 and are associated with resistance to mAbs T10C9, NB3C4 and T1E7 respectively. The three epitopes are within a region of the primary polypeptide sequence of E2 bounded by the two glycosylation sites at amino acids 200 and 262 (Dalgarno *et al.*,1983) and are defined by residues 216 (a), 232, 234 (b1) and 246, 248, 251 (b2). A multiepitope variant resistant to all three mAbs was also selected (Vrati *et al.*,1988).

As the three dimensional structure of E2 has not been resolved it is not possible to specify the spatial relationship of these three epitopes. Although RRV variants resistant to one mAb are neutralized normally by the other two, the mAbs compete with each other in CBAs. This suggests that the epitopes are functionally independent but topographically close. It has been proposed that these epitopes plus another, epitope c, which has

been defined by CBAs and which interacts with epitope b, represent separate but interacting parts of a single, major neutralizing determinant on E2 (Vrati *et al.*,1988).

To extend this work two questions were asked: (1) Are epitopes a, b1 and b2 significant in a normal polyclonal immune response, and if so is any one epitope dominant? (2) Are synthetic peptides representing the primary amino acid sequence around the neutralization epitopes able to elicit neutralizing antibodies against RRV and bind to anti-RRV antibodies?

Synthetic peptides have been used to define antigenic regions of a number of viruses important in human and animal health, eg poliovirus (Emini *et al.*,1983), foot and mouth disease virus (Bittle *et al.*,1982; Pfaff *et al.*,1982; Geysen *et al.*,1984, 1985; Meloen *et al.*,1987), influenza virus (Green *et al.*, 1982; Shapira *et al.*,1984), human immunodefficiency virus (Kennedy *et al.*,1986), Human T cell leukaemia virus Type I (Copeland *et al.*,1986) and feline leukaemia virus (Elder *et al.*,1987). For polio and foot-and-mouth disease viruses, synthetic peptides induce antibodies which neutralize virus and protect animals from challenge (Bittle *et al.*,1982; Emini *et al.*,1983; DiMarchi *et al.*,1986). Such peptides have the potential to form the basis of safe synthetic vaccines (Arnon, 1986). This approach has not been used to examine the antigenic structure of an alphavirus.

In this chapter, the importance of the defined neutralization determinants of RRV E2 in a polyclonal immune response has been examined. In addition studies have been made of the antigenicity and immunogenicity of four synthetic peptides representing the primary amino acid sequences around epitopes a, b1 and b2. It is concluded that a substantial proportion of the neutralizing antibodies in a polyclonal

hyperimmune response recognize the combination of these epitopes and that the epitopes are probably conformationally discontinuous.

2.2 Materials and Methods

2.2.1 Virus stocks

The prototype T48 strain of RRV (Doherty *et al.*,1963a) was from the Yale Arbovirus Research Unit (New Haven, Conn. USA). It had been passaged approximately ten times in mice when received. The NB5092 strain of RRV (Gard *et al.*,1973) was from Dr.I. D. Marshall (John Curtin School of Medical Research, Australian National University, Canberra, Australia). Virus was plaque purified on Vero cell monolayers and working stocks were prepared in BHK cells. Virus was assayed by plaque formation on Vero cell monolayers (Newton *et al.*,1981) and titres are expressed as plaque forming units per ml (pfu/ml).

The mAb resistant variants of RRV T48 used are given below, with the amino acid change, position in E2 and epitope in brackets: Tv42 (Thr216→Ile, a), Tv61 (Lys234→Gln, b1), Tv1 (Asp246→Asn, b2). These variants were selected with mAbs T10C9, NB3C4 and T1E7 respectively. Tv161 (Thr216→Ile, a; Lys234→Asn, b1; Asp246→Asn, b2) was selected sequentially with all three mAbs (Vrati *et al.*,1988). Stocks of these variants were grown in BHK cells and assayed for mAb resistance, at a 1:50 mAb dilution, before use.

2.2.2 Cells

BHK monolayers were grown in Glasgow Minimum Essential Medium (GMEM) supplemented with 8% bovine serum (BS). Vero cells

were grown in Medium 199 enriched with lactalbumin hydrolysate (M199 LAH) plus 10% BS. Cell cultures were incubated at 36° in 5% CO_2.

2.2.3 Virus purification

This essentially followed the method of Ou *et al.* (1981). Two roller bottles of BHK cells were infected with RRV (moi~0.25). Four hours post infection (pi) one roller bottle was pulsed with 5µg/ml of actinomycin D (AMD; Merck, Sharpe and Dohme, NJ, USA) for 30 min. Following this, the growth medium was removed and the monolayers washed twice with Hank's balanced salt solution (HBSS). Medium was replaced with Eagle's minimal essential medium (EMEM) containing 2% dialysed foetal calf serum (FCS) and 5µCi/ml [5-^{3}H]-uridine (NEN Research Products, Boston, MA, USA). Supernatants were harvested 24 hours post-infection and pooled. Following clarification (10,000 rpm, 10 min, 4°), virus was precipitated by addition of one-fourth volume of 40% polyethylene glycol (PEG; Koch-Light Laboratories, UK) in HBSS (no BSA) and mixing for 2 hours at 4°. PEG precipitates were collected by centrifugation (Sorvall GSA rotor, 10,000 rpm, 50 min). Pellets were resuspended in 4 to 5 ml of NET buffer (0.15M NaCl; 0.01M Tris, pH7.5; 0.002M EDTA) and centrifuged (27,000 rpm, 90 min) through a 5-25% sucrose gradient in NET. Peak gradient fractions were identified by liquid scintillation counting, pooled and stored at -70°.

2.2.4 Antisera

Polyclonal antiserum was produced in Balb/c mice (6-8 weeks old), immunized intraperitoneally (ip) with 10^6 pfu of sucrose gradient purified RRV T48 without adjuvant. Mice were boosted ip with the same dose of virus at 21 days and again 14 days later. Blood was collected from the orbital plexus into heparinized capillary tubes (Monoject Scientific, St Louis MO, USA); plasma was separated by centrifugation and stored at

-20°. [Balb/c mice were used because the mAbs described by Vrati *et al.* (1988) were produced in this breed]. Other polyclonal antibodies were produced as ascitic fluid in outbred Walter and Elisa Hall Institute mice (Tikasingh *et al.*, 1966). Polyclonal rabbit anti-RRV serum was a gift from Dr. I.D. Marshall. The immunoglobulin fraction was concentrated from this serum by ammonium sulphate precipitation.

Monoclonal antibodies T1E7, T10C9 and NB3C4 (Vrati *et al.*,1988) were used as high titre mouse ascitic fluid.

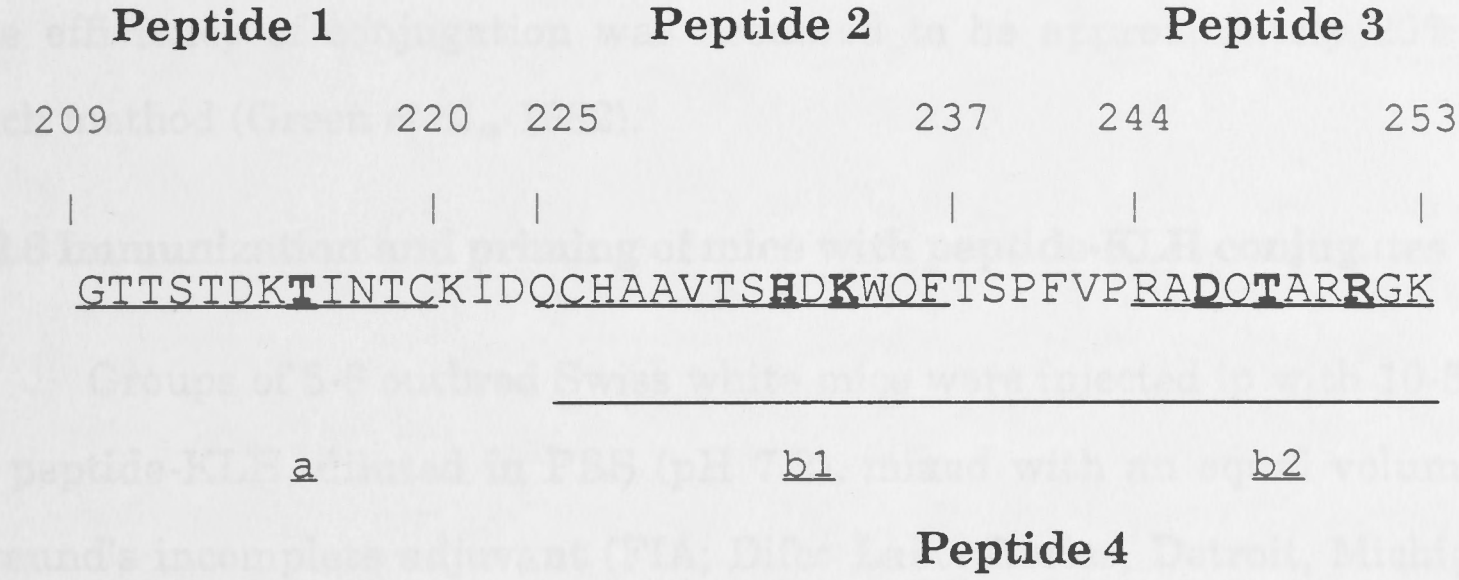
2.2.5 Synthetic peptides and conjugation to carrier proteins

Synthetic peptides were prepared by Dr. G. W. Tregear, Howard Florey Institute, University of Melbourne, Parkville, Victoria. The sequence and position of the peptides in the primary amino acid sequence of RRV E2 is shown in Fig. 2.1. Peptides 1, 2 and 4 were conjugated through their terminal cysteine residues to bovine serum albumin (BSA; Sigma, St Louis, MO, USA) or keyhole limpet haemocyanin (KLH; Sigma) using the heterobifunctional linker m-maleimidobenzoyl-N-hydroxysuccinimide (MBS; Boehringer Mannheim, West Germany) (Liu *et al.*,1979; Green *et al.*, 1982). 4mg KLH or BSA in 0.25ml 10mM phosphate buffer (pH 7.0) was reacted with 0.7mg MBS (dissolved in dimethyl formamide, 24.8mg/ml) and stirred for 30 min at 37°. The reaction mixture was passed down a Sephadex G-25 column equilibrated with 50mM phosphate buffer (pH 6.0) to remove free MBS. The KLH-MB or BSA-MB was then incubated with 5mg of peptide in phosphate buffered saline (PBS, pH 7.2) at room temperature and the pH adjusted to 7.0-7.5 with 1M KOH. The mixture was stirred at room temperature for three hours, divided into aliquots and stored at -20°.

Peptides 1, 2 and 3 were also coupled (through free amino residues) to KLH or BSA using glutaraldehyde (Reichlin, 1980). Peptide (1mg) in 10-

Figure 2.1

Location of synthetic peptides and neutralization epitopes on glycoprotein E2 of RRV T48



The amino acid sequence of the E2 glycoprotein of RRV T48 from residue 209 to 253 is shown. Residues are numbered from the N-terminus of E2 (Dalgarno *et al.*, 1983). Synthetic peptide sequences are underlined. Amino acids defining neutralization epitopes a, b1 and b2 (Vrati *et al.*,1988) are in bold type.

20µl PBS (pH 7.2) was mixed with 5mg of KLH or 7mg of BSA in 1ml of PBS. Glutaraldehyde (21mM) in PBS (0.5 ml) was added dropwise over 30-40 min with constant mixing and the reaction allowed to proceed at room temperature overnight, before dialysing against PBS. The conjugate was divided into aliquots and stored at -20°. For estimating immunizing doses the efficiency of conjugation was assumed to be approximately 20% for each method (Green *et al.*, 1982).

2.2.6 Immunization and priming of mice with peptide-KLH conjugates

Groups of 5-6 outbred Swiss white mice were injected ip with 10-50µg of peptide-KLH, diluted in PBS (pH 7.2), mixed with an equal volume of Freund's incomplete adjuvant (FIA; Difco Laboratories, Detroit, Michigan) in a final volume of 100 µl. Mice were bled before and, at seven day intervals, after immunization. Boosting was with the same dose of peptide-KLH in FIA 28 days after the primary immunization and again at 14 to 21 day intervals to a maximum of four boosts. Titres did not increase after the second boost. To examine the necessity for conjugation, one group was immunized with free peptide 4 (10µg/mouse; in PBS, 1:1 with FIA in a total volume of 100 µl). For convenience each group of mice was allocated an alphabetical code letter. The groups of mice, immunogen and method of coupling to KLH are summarized in Table 2.1.

To assay for priming by peptides (see results), mice in groups B and E were divided into two sub-groups 28 days after the primary immunization; one sub-group was boosted with the appropriate peptide-KLH conjugate and the other with the equivalent of 10^6 pfu of UV-inactivated RRV T48. Mice in groups H and J were treated in the same fashion but after hyperimmunization (three boosts). Control mice (no previous immunization) were injected with the equivalent of either 10^6 or 10^7 pfu of UV-inactivated RRV T48. Sera from boosted or control mice were

Table 2.1

Immunization of mice with synthetic peptides*

Group	No. of mice	Immunogen	Coupling agent
B	5	Peptide 1-KLH	MBS
C	6	Peptide 1-KLH	Glutaraldehyde
D	6	Peptide 2-KLH	Glutaraldehyde
E	6	Peptide 3-KLH	Glutaraldehyde
H	6	Peptide 2-KLH	MBS
J	6	Peptide 4-KLH	MBS
K	6	Peptide 4	-

*For immunization 10-50μg of peptide or peptide-KLH was injected into mice 8-12 weeks old. The peptide or peptide-KLH was diluted in PBS and mixed with an equal volume of FIA to give a final injection volume of 100μl.

collected five, seven or ten days after injection and examined for RRV binding in ELISA and neutralization assays.

2.2.7 Enzyme linked immunosorbent assays (ELISA)

Direct ELISA. Antigen (purified RRV, conjugated or free peptide, BSA or KLH) was adsorbed to round-bottomed, 96-well, polyvinylchloride (PVC) microtitre trays (Titertek, Flow Laboratories) by adding 25µl of an appropriate dilution to each well, incubating at 37° for 2 hours and leaving at 4° overnight. All dilutions were in PBS (pH 7.2). Trays were washed with 5% skim milk powder in PBS (milk/PBS) to remove unadsorbed antigen and to block the wells. Antiserum diluted in 5% milk/PBS was added and incubated for two hours at 37°. Wells were washed with Tween 20 (0.02% in PBS; Sigma) and 25µl of goat anti-mouse immunoglobulin conjugated to horse radish peroxidase (1:600 in milk/PBS; Bio-Rad, Richmond, CA, USA) was added. Incubation was for 30 min at 37°; the wells were washed with Tween 20 as above and 100µl of ABTS (2,2-Azino bis 3-ethylbenzthiazoline sulphonate; Boehringer Mannheim) 1mg/ml in 0.1M citrate phosphate buffer (pH 4.0), hydrogen peroxide (0.006%) was added to each well. Trays were incubated for 15 min at room temperature; OD (410nm) was determined with a Dynatech MicroELISA II automated plate reader.

Capture ELISA. Rabbit anti-RRV immunoglobulin (1:2000 in PBS) was adsorbed to PVC microtitre trays (50µl/well). After washing and blocking with milk/PBS, purified RRV T48 was added (50µl/well) and the trays incubated at 37° for two hours. Wells were washed with milk/PBS and antiserum (50µl per well) added. After two hours incubation, antibody binding was determined as described for the direct ELISA. The assay was standardized such that polyclonal antiserum to RRV had the same titre in the capture ELISA as in the direct ELISA. Monoclonal antibodies T10C9,

T1E7 and NB3C4 also bound to RRV with similar or slightly higher titres in the capture ELISA compared to the direct assay.

2.2.8 Competition binding assays

An approximately 10^5-fold molar excess of peptide was incubated (60 min, 37°) with a concentration of mAb chosen to be within the linear region of the antibody-virus binding curve in ELISA. Controls consisted of the same amount of mAb incubated with either PBS or purified virus. The peptide-mAb mixture or the controls were assayed in an ELISA for binding to RRV as measured by the OD (410nm); assays were in quadruplicate. Inhibition of binding was estimated by the OD obtained with mAb plus peptide compared with the OD obtained with mAb plus PBS.

2.2.9 Plaque reduction neutralization assays (PRNA)

Approximately 100-200 pfu of virus (120µl in HBSS, pH 7.2) was incubated (60 min, 37°) with 120 µl of monoclonal or polyclonal antibodies diluted in HBSS, or with anti-peptide sera and assayed, in duplicate, for infectivity by plaque formation on Vero cell monolayers. Percent plaque reduction was calculated relative to virus controls incubated with normal mouse serum or ascitic fluid. Antibody titres were expressed as the reciprocal of the dilution producing a fifty percent reduction in plaque numbers. This dilution was estimated by interpolation.

2.2.10 Immunoprecipitation of RRV by anti-peptide sera

[5-^{3}H]-uridine-labelled RRV T48, (~3000 cpm in 20µl PBS, representing ~450ng of viral protein, estimated by Bio-Rad protein assay), prepared as described in 2.2.3, was mixed with an equal volume of mouse antiserum or normal mouse serum and incubated at 37° for 60 min. 50µl of a 10% (w/v) suspension of washed, formalin-fixed *Staphylococcus aureus* cells (Calbiochem, La Jolla, CA, USA.) was added and the mixture

incubated (37°, 60 min) with frequent mixing. Cells were pelleted by centrifugation, washed and resuspended twice before precipitation with 5% trichloracetic acid (TCA) on 20mm glassfibre discs. Radioactivity was determined in a Beckman liquid scintillation counter. Washing and resuspension were in TNA (NaCl, 0.2M, Tris pH 7.5, 0.05M, EDTA, 1mM, 0.1%BSA, 0.5% Triton-X 100).

Titration with polyclonal anti-RRV in the immunoprecipitation assay indicated that this system was of relatively low sensitivity compared to the ELISA, where the same antiserum had a ten fold higher titre (data not shown), although the antigen was the same for both. This low sensitivity was probably due to the low specific activity of the labelled virus:~1 cpm / 1000 pfu.

2.3 Results

2.3.1 Neutralization of RRV variants by anti-RRV T48-sera

It has been postulated that epitopes a, b1 and b2 form part of a major neutralization domain on RRV E2 (Vrati *et al.*,1988). To analyse the contribution of these epitopes to neutralization in a polyclonal immune response, antiserum was prepared against RRV T48 in five Balb/c mice (Methods; 2.2.4). Using PRNAs, sera were titrated against the following mAb resistant RRV T48 variants: Tv1 (epitope b2), Tv42 (epitope a), Tv61 (epitope b1), and Tv161 (epitopes a, b1 and b2) and against RRV NB5092. NB5092 has five amino acid differences from T48 in E2 (Faragher *et al.*, 1988); one of these, at position 251, is within epitope b2 and is probably responsible for the difference in neutralization of these two strains by mAb T1E7 (Vrati *et al.*,1988).

RRV variants with amino acid changes in single epitopes (ie Tv1, Tv42, Tv61 and NB5092) could not be differentiated from T48 in neutralization assays (Table 2.2). However, a consistent difference in neutralization titre was obtained between T48 and Tv161 using sera collected from mice after the second boost, with differences in the 50% neutralization point of 3 to 8 fold (Fig 2.2). These assays were repeated four times with similar results. Similar differences were obtained with sera from the same mice following a third boost and titration of this serum from mouse 3 is shown in Fig 2.2. Sera collected at days seven and fourteen after the primary immunization showed no differences in neutralization titre between RRV T48 and Tv161 in PRNA using two-fold dilutions across a range of 90% to less than 20% neutralization (data not shown). Sera from the five mice titrated independently gave similar results.

2.3.2 Studies with synthetic peptides

It appeared from the results above that the region defined by epitopes a, b1 and b2 is significant in neutralization and could be a potential candidate for a synthetic vaccine. As the three dimensional structure of E2 has not been determined, the structure of the epitopes and the surrounding regions is undefined. If there were epitopes with significant continuous structure in this area they might be mimicked by synthetic peptides. To investigate the antigenicity and immunogenicity of this region, four peptides representing the primary amino acid sequences around epitopes a, b1 and b2 (Fig 2.1) were synthesized, coupled to KLH and used to immunize groups of mice (Table 2.1).

Table 2.2

Neutralization of RRV mAb escape variants and RRV NB5092 by polyclonal antisera to RRV T48[1]

RRV strain	Mouse number				
	1	2	3	4	5
T48	3.1	3.9	4.1	3.1	3.6
Tv1	3.0	3.7	3.9	3.3	3.5
Tv42	3.2	3.7	4.0	3.1	3.4
Tv61	3.3	3.8	3.9	3.0	3.5
NB5092	3.1	3.9	4.0	3.0	3.5

[1]The neutralization titre, for each virus with serum from each mouse, is shown as the reciprocal of the $\log_{10}$ dilution of antiserum causing 50% neutralization of virus plaque formation. Two-fold dilutions of antisera were used and the 50% endpoints were estimated by interpolation. Assays for all five viruses were performed in parallel.

Figure 2.2

Neutralization of RRV T48 and Tv161 by polyclonal anti-T48-sera

Neutralization of RRV T48 and Tv161 was measured by PRNA using polyclonal antisera raised against RRV T48. Sera raised in five Balb/c mice were titrated independently. Approximately 220 pfu of each virus were incubated with each dilution of serum for 60 min at 36°. Controls were incubated with 1:400 normal serum. Duplicate 100µl aliquots of each incubation were plaque assayed on Vero cell monolayers and the percentage neutralization at each dilution calculated as the reduction in mean plaque numbers compared to the controls.

The percent neutralization of T48 (solid line) and Tv161 (broken line) by serum collected from each mouse seven days after the second boost are shown for serum dilutions between 1:400 and 1:6400 (panels A-E). Panel F shows the titration of serum from mouse 3 collected seven days after the third boost.

A mouse 1

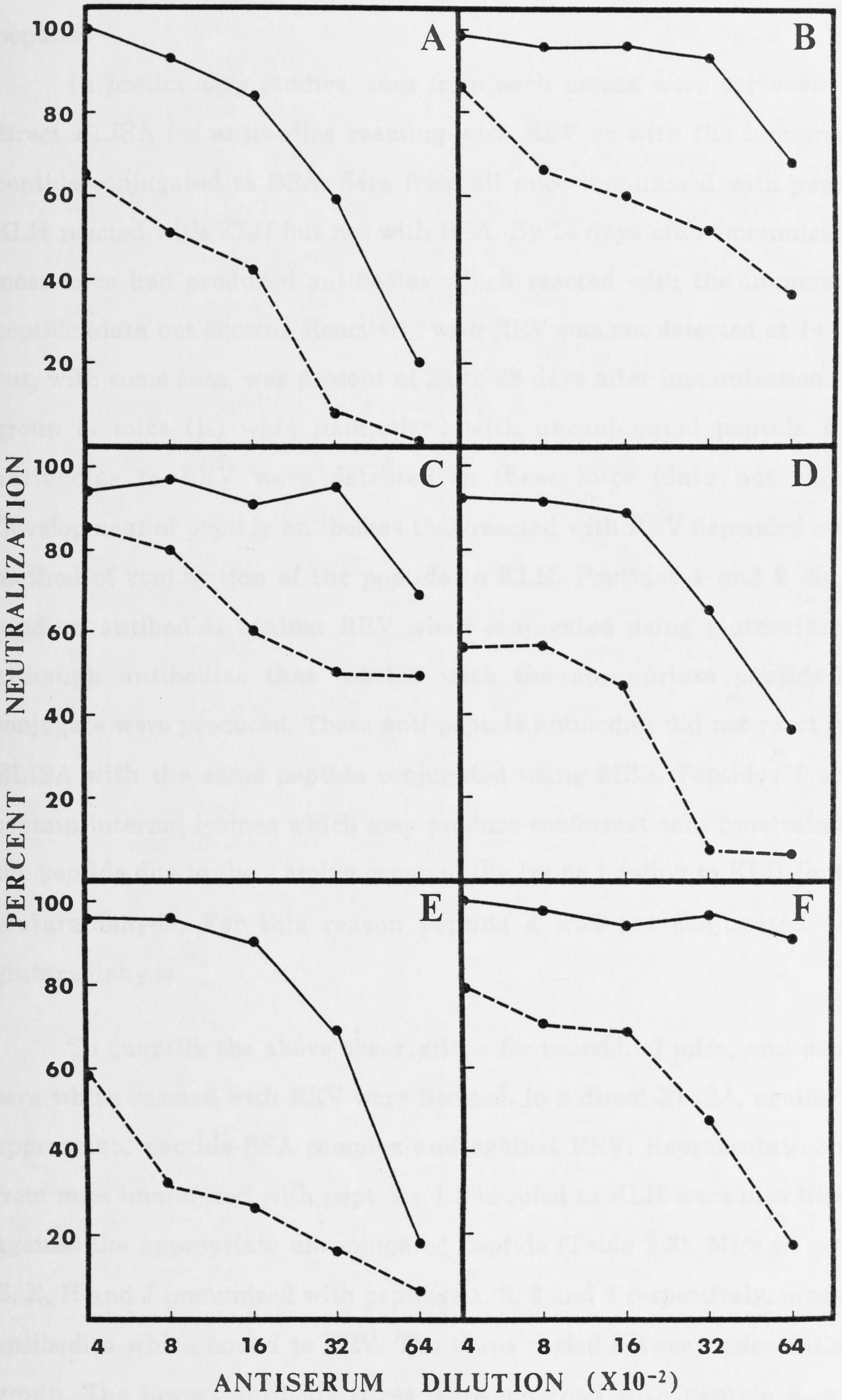
B mouse 2

C mouse 3

D mouse 4

E mouse 5

F mouse 3, third boost


A
B
C
D
E
F
100
80
60
40
20
PERCENT NEUTRALIZATION
4
8
16
32
64
ANTISERUM DILUTION (X10⁻²)

2.3.3 Direct ELISA titrations of sera from mice immunized with synthetic peptides

In preliminary studies, sera from each mouse were screened in a direct ELISA for antibodies reacting with RRV or with the immunizing peptide conjugated to BSA. Sera from all mice immunized with peptide-KLH reacted with KLH but not with BSA. By 14 days after immunization, most mice had produced antibodies which reacted with the immunizing peptide (data not shown). Reactivity with RRV was not detected at 14 days but, with some sera, was present at 21 to 28 days after immunization. One group of mice (K) were immunized with unconjugated peptide 4; no antibodies to RRV were detected in these mice (data not shown). Development of peptide antibodies that reacted with RRV depended on the method of conjugation of the peptide to KLH. Peptides 1 and 2 did not produce antibodies against RRV when conjugated using glutaraldehyde although antibodies that reacted with the appropriate peptide-BSA conjugate were produced. These anti-peptide antibodies did not react in an ELISA with the same peptide conjugated using MBS. Peptides 1 and 2 contain internal lysines which may produce conformational constraints on the peptide due to the ε amino group of the lysine binding to KLH through glutaraldehyde. For this reason peptide 4 was not conjugated using glutaraldehyde.

To quantify the above observations for individual mice, anti-peptide sera which reacted with RRV were titrated, in a direct ELISA, against the appropriate peptide-BSA complex and against RRV. Representative sera from mice immunized with peptides 1-3 coupled to KLH were also titrated against the appropriate unconjugated peptide (Table 2.3). Mice in groups B, E, H and J immunized with peptides 1, 2, 3 and 4 respectively, produced antibodies which bound to RRV. The titres varied between mice within a group. The lowest antibody titres were obtained with peptide 3, while

Table 2.3

[1]Sera were tested in ELISA for RRV and peptide binding. In these assays the end point was taken as being 0.1 OD units above the controls. Shown are maximum titres (irrespective of the time of harvest), measured as the reciprocal of the end point dilution, in a direct ELISA using the indicated antigens. Data is only shown for those groups of mice which produced antibodies which reacted with RRV

ND not determined.

* binding below this dilution was not examined.

† number indicates the identity of the mouse within the group.

Table 2.3

ELISA titres of sera from mice immunized with synthetic peptides [1]

			ELISA titre against indicated antigen		
Group	Immunogen	Mouse no.†	RRV	Peptide-BSA	Peptide
B	PEP 1-KLH	1	20480	64000	ND
		4	2560	64000	4000
		5	5120	32000	ND
H	PEP 2-KLH	1	1280	64000	ND
		2	640	64000	ND
		3	<10	64000	ND
		4	>1280	64000	16000
		5	320	64000	ND
		6	160	64000	ND
E	PEP 3-KLH	4	640	8000	<500*
		5	40	ND	ND
		6	80	32000	ND
J	PEP 4-KLH	1	2560	ND	ND
		2	2560	ND	ND
		3	>2560	ND	ND
		4	2560	ND	ND
		5	2560	ND	ND
		6	>2560	ND	ND

peptide 4 produced the most consistent response. When unconjugated peptides were used as antigen in the ELISA, titres were 4-16 fold lower than those with peptide-BSA (Table 2.3).

2.3.4 Amino acid sequence specificity of anti-peptide sera

To determine the specificity of E2 sequence recognition of the anti-peptide sera, representative sera from mice immunized with peptides 1, 2 or 3 coupled to KLH, were titrated against RRV T48 and against variants Tv1, Tv42 and Tv61 in a direct ELISA (Table 2.4). Anti-peptide 1 serum had the same reactivity towards T48, Tv1 and Tv61 but had a greater than four fold reduction in titre against Tv42 which has an amino acid alteration in the peptide 1 sequence (Thr216→Ile). Antiserum to peptide 2 reacted with T48, Tv1 and Tv42 but did not bind to Tv61 which has a change in the peptide 2 sequence (Lys234→Gln). Peptide 3 antiserum reacted with T48, Tv42 and Tv61 but not with Tv1 which has an alteration in the peptide 3 sequence (Asp246→Asn). These results demonstrate the sequence specificity of the binding of the anti-peptide sera to the region of RRV E2 represented by the immunizing peptide; it is also clear that this region must be exposed under the conditions of the assay.

2.3.5 Examination of anti-peptide sera for neutralizing activity

To determine whether antibodies against the synthetic peptides neutralized RRV, the sera were tested in PRNA. No neutralizing activity was demonstrated at dilutions as low as 1:2 with sera collected from primary immunizations or after multiple boosts. The addition of complement had no effect (data not shown).

Table 2.4

The specific binding of anti-peptide antibodies to RRV T48 and the variants Tv1, Tv42 and Tv61 in a direct ELISA[1]

	Virus			
Antisera	T48	Tv1	Tv42	Tv61
Peptide 1	>1280	>1280	320	>1280
Peptide 2	>1280	>1280	>1280	<20
Peptide 3	160	<20	320	320

[1]Sera from mice immunized with peptides 1, 2 or 3 coupled to KLH and which reacted with RRV T48 in a direct ELISA were tested for binding to Tv1, Tv42 and Tv61 in a direct ELISA. Twofold serum dilution intervals were used; titres are the reciprocal of the end point dilutions, where the end point was taken as being 0.1 OD units above the controls.

2.3.6 Mice immunized with synthetic peptides were not primed to respond to a sub-immunogenic dose of RRV

Work with poliovirus (Emini *et al.*,1983) and cholera toxin (Jacob *et al.*,1986) has shown that synthetic peptides which fail to induce neutralizing antibodies can prime animals for a neutralizing response when the immunized animal is exposed to an otherwise sub-immunogenic dose of virus or toxin. To examine whether mice immunized with RRV peptides were primed for an immune response to RRV, two mice immunized with peptide 1-KLH (from group B) and three immunized with peptide 3-KLH (from group E) (Table 2.1) were injected, 28 days after primary peptide immunization, with the equivalent of 10^6 pfu of UV-inactivated RRV T48. The remaining three mice in each group were boosted with peptide-KLH. Unprimed control mice were injected with the equivalent of 10^6 or 10^7 pfu of UV-inactivated RRV. All mice were bled five and ten days after injection. No neutralizing activity was detected in mice boosted either with peptides or inactivated virus nor in control mice injected with 10^6 pfu of inactivated RRV. Mice in group B boosted with peptide had a 4-32 fold rise in ELISA titre against RRV; the two mice boosted with inactivated RRV gave no rise in titre. Control mice injected with 10^7 pfu of inactivated RRV produced antibodies which reacted with RRV in an ELISA; these were not tested for neutralization. Mice were also tested for priming by injection of the equivalent of 10^6 pfu of inactivated RRV after multiple immunizations with peptides 2 or 4. None of the sera tested neutralized RRV at a 1:2 serum dilution (data not shown). It was concluded that mice immunized with peptides 1,2, 3 or 4 were not primed to produce a neutralizing response to small doses of inactivated RRV.

2.3.7 Immunoprecipitation of RRV

Because antibodies from mice immunized with peptides 1-4 bound to RRV in an ELISA but did not neutralize the virus the following question arises: does RRV adsorbed to a PVC solid phase bind antibodies differently to free virus? To examine the binding of anti-peptide antibodies to RRV in solution, sera from mice immunized with peptides 1, 2, 3 or 4, conjugated to KLH, were tested for their ability to bind ^{3}H-uridine labelled RRV in an immunoprecipitation assay (Methods; 2.2.10). Only 3% of virus was precipitated in the presence of anti-sera against peptide 1, 2 or 3, the same as with normal serum. Anti-peptide 4 sera precipitated an average of 25% of virus. Polyclonal anti-serum against RRV T48 precipitated 88% of virus (data not shown). Thus only anti-peptide 4 serum was able to bind significantly to free RRV.

2.3.8 Binding assays of anti-peptide sera in capture ELISA

Direct adsorption of viruses to PVC in an ELISA creates the potential for partial denaturation of viral proteins and the exposure of cryptic epitopes (McCullough *et al.*,1985; Olmsted *et al.*,1986). To determine whether cryptic epitopes recognized by anti-peptide sera were exposed when RRV was bound directly to the ELISA trays, a capture ELISA was developed (Methods; 2.2.7). This assay avoided direct adsorption of RRV to a plastic surface by coating the wells with rabbit anti-RRV antibodies. Added virus was then bound by these antibodies, and mouse anti-peptide or anti-RRV sera were assayed for binding to the "captured" virus.

Anti-peptide sera to peptides 1, 2, 3 or 4 which reacted with RRV in a direct ELISA, were assayed for binding to RRV in a capture ELISA; no sera bound to RRV at dilutions down to 1:10 (data not shown). This assay was repeated several times with the same result. Control anti-RRV

polyclonal and monoclonal antibodies bound to RRV with similar titres in the capture assay and in the direct assay. It was concluded that the adsorption of RRV to a plastic surface distorted E2 and exposed epitopes specifically recognized by antibodies raised to peptides from the corresponding regions.

2.3.9 Testing the antigenicity of synthetic peptides

In addition to being potential immunogens, synthetic peptides have been used to define continuous antigenic sites on proteins by their ability to bind monoclonal and polyclonal antibodies raised against the intact protein (reviewed by Van Regenmortel, 1989). To examine whether antibodies raised against RRV could bind to the synthetic peptides, mAbs T10C9, NB3C4 and T1E7 and polyclonal antiserum were assayed, in an ELISA, for binding to unconjugated peptides 1, 2 and 4 or to peptides 1, 2 and 3 coupled to KLH or BSA. None of the antisera bound to KLH or BSA; mAb T1E7 did bind to peptide 3-KLH at high antibody concentrations but only marginally above the background binding to the KLH-glutaraldehyde control. There was no significant binding of the other antibodies to any of the peptides or peptide conjugates tested (data not shown).

2.3.10 Competition binding assays

Peptides bound to a solid phase as in the ELISA may not be able to adopt an appropriate conformation for antibody recognition (Van Regenmortel, 1989). To examine whether antibodies to RRV bound peptides in solution, the binding of peptides 1, 2, 3 and 4 to mAbs T10C9, NB3C4 and T1E7 was tested in competition binding experiments (Methods; 2.2.8). A large excess of peptide was incubated with a low concentration of mAb, chosen to be in the linear region of the mAb/RRV binding curve in an ELISA, so that changes in free antibody concentration should be readily apparent. Samples were assayed for binding to RRV in an ELISA. There

was no inhibition of binding to RRV of any of the mAbs with any of the peptides. However, whole virus inhibited 90% of T1E7 and NB3C4 binding and 70-80% of T10C9 binding in this assay system.

2.4 Discussion

2.4.1 Characterization of a major neutralization domain of RRV

Neutralization assays using polyclonal antisera to RRV T48 suggest that a significant proportion of the neutralizing antibodies produced in hyperimmune mice are directed to the region on E2 defined by epitopes a, b1 and b2. Hyperimmune sera from five individual mice showed a 3-8 fold decrease in 50% neutralization titre against the triple epitope variant, Tv161, compared to T48 indicating that the altered site was poorly recognized by a proportion of the neutralizing antibodies. Variants changed at single amino acids showed no difference in neutralization titres compared to RRV T48. Thus no single epitope is immunodominant but the sum of these determinants comprises a detectable neutralization domain.

RRV NB5092 has five amino acid changes from T48 in E2 and three in E1 (Faragher *et al.*,1988). As previously reported (Gard *et al.*,1973) and confirmed here, it is neutralized to the same extent as RRV T48 by polyclonal anti-T48-sera. Only one of the amino acid differences in E2 (residue 251 in epitope b2) is within an established neutralization epitope. This may be responsible for the resistance of this isolate to mAb T1E7 (Vrati *et al.*,1988).

Similar results have been found with SIN. Variants selected sequentially for resistance to three mAbs, two with epitopes on E2 and one which binds to E1 were neutralized less efficiently by polyclonal serum

raised against the parental virus (Stec *et al.*,1986). When a variant of SIN selected for rapid growth in BHK cells was compared with the parental virus in polyclonal neutralization tests, both viruses were neutralized equally well by antiserum to the parental virus, however, the parental strain was only marginally neutralized by antiserum against the variant (Baric *et al.*,1981). This variant has a single amino acid change (Ser114→Arg) in E2 which may cause a significant conformational change in the surface of E2 (Davis *et al.*,1986).

In viruses other than alphaviruses an amino acid change in a single epitope can significantly alter the reactivity of the virus with polyclonal antibodies. This may vary in the course of the immune response and be dependent on the species and the individual animal in which the antiserum is raised. For example, six out of ten mAb selected variants of influenza B virus were significantly different from the parent virus using primary ferret antisera, with differences in titre of 4-16 fold (Webster and Berton, 1981). When the comparison was made using goat hyperimmune serum only three of the variants had four fold differences in titre and one had a two-fold difference from the parent virus. On the other hand most mAb resistant variants of influenza A virus could not be distinguished from the parental virus using polyclonal antisera (Laver *et al.*,1979; Webster and Laver, 1980; Gerhard and Webster, 1978). Monoclonal antibody resistant variants of vesicular stomatitis virus altered at single epitopes (A or B) could be distinguished from the parent virus using hyperimmune serum with differences of 3-9 fold in titre (Lefrancois and Lyles, 1983). When primary sera were used, larger differences (30-70 fold) were observed between the epitope A variants and the wild-type. However, the epitope B variant could not be differentiated from the wild-type. A mAb resistant variant of a human rotavirus had a greater than ten fold increase in resistance to polyclonal hyperimmune serum compared with the parent

virus (Dyall-Smith *et al.*,1986). Because hyperimmune serum would be expected to contain neutralizing antibodies to many different determinants it was postulated that a single amino acid change (leading to a new potential glycosylation site) altered an immunodominant antigenic site.

Significant changes in neutralization due to alterations at a single epitope could be important in vaccine production where a vaccine might be rendered ineffective by a single amino acid change in a critical antigenic determinant. Two diverged isolates of RRV, T48 and NB5092, appear not to have changed substantially in neutralization epitopes, furthermore single epitope variants of T48 were not detectably different from the parent virus in neutralization by primary or hyperimmune sera. This suggests that a vaccine for RRV would give wide protection, covering existing and evolving strains of the virus.

Epitopes a, b1 and b2 are defined by amino acids 216(a), 232 and 234(b1) and 246, 248 and 251(b2) on RRV E2. Thus these epitopes are very close together in the primary polypeptide sequence. Although the three dimensional structure of E2 has not been resolved, several interesting features of this region can be defined from the primary amino acid sequence (Dalgarno *et al.*,1983). It is bounded by glycosylation sites at amino acids 200 and 261; it is an extremely hydrophilic region with hydrophilic spikes at epitopes b1 and b2 and there are four cysteine residues, strongly conserved between alphaviruses. These could be involved in disulphide bonds and determine protein folding and stability. The hydrophilicity plus the presence of the glycosylation sites indicate that this region is likely to be on the surface of the E2 protein, as would be anticipated from the presence of the mAb epitopes. Sites outside this region of the primary sequence are also involved in neutralization of RRV; a deletion at amino acids 55-61 (Vrati *et al.*,1986) affects epitope b1,

suggesting that these regions of the primary polypeptide may fold into apposition in the tertiary structure.

2.4.2 Synthetic peptides as immunogens and antigens

Linear regions of E2 containing neutralization epitopes could potentially form the basis of synthetic peptide vaccines for RRV. This was investigated for epitopes a, b1 and b2 using four synthetic peptides covering most of the amino acid sequence from residues 209 to 253 of E2. Peptides coupled to KLH were strongly immunogenic in mice and anti-peptide sera specifically recognized RRV in a direct ELISA. However, anti-peptide antibodies did not neutralize RRV nor did they bind to RRV in a capture ELISA.

It appears that the reactivity of the anti-peptide antibodies with RRV was dependent on partial denaturation of the virus when it was bound to a plastic surface. Thus anti-peptide sera did not neutralize RRV nor did they bind to the virus in a capture ELISA. In immunoprecipitation experiments antibodies to peptide four (the longest of the peptides) precipitated 25% of added virus but antisera to the other three peptides did not precipitate RRV. As there was no binding in the capture ELISA, it is possible that partial denaturation of a proportion of the virus had occurred during viral purification, leading to recognition by anti-peptide four serum; this denatured virus may not have been bound in the capture ELISA. Denaturation of viral antigen on binding to a solid phase has been described for SIN and foot-and-mouth disease virus (FMDV) (Olmsted *et al.*,1986; McCullough *et al.*,1985).

Monoclonal or polyclonal antisera to RRV did not bind to either free or conjugated peptide. This indicated that either the peptides did not contain enough amino acids involved in antibody binding or that the peptides were unable to present these residues in an appropriate

conformation. It was concluded that the peptides examined did not mimic significant portions of antigenic sites. This, together with the failure of anti-peptide sera to bind to native RRV, suggests that the neutralization epitopes in the region of E2 between amino acids 209 and 253 are discontinuous with secondary or tertiary folding of the primary polypeptide sequence bringing critical amino acids together.

The complexity of predicting whether peptides can mimic antigenic sites can be seen by considering the examples of influenza virus and FMDV. For FMDV, antibodies raised to peptides representing a prominent antigenic loop at amino acids 133 to 158 of VP1 neutralize the virus and protect animals from challenge (Bittle *et al.*,1982; DiMarchi *et al.*,1986). Acharya *et al.* (1989) reported the crystallographic structure of FMDV and suggested that the success of this region in producing peptide immunogens probably relates to an unusual combination of marked protrusion of the loop and very limited structural constraints for antibody recognition. On the other hand, for influenza virus, antigenic site A is formed by a prominent loop in the region of 140 to 146 of the three dimensional structure of the haemagglutinin molecule (Wiley *et al.*,1981). Although this is a target for neutralizing antibodies and undergoes antigenic drift (Air and Laver, 1986), peptides corresponding to this region have not produced antibodies that react with native virus (Jackson *et al.*,1982; Shapira *et al.*,1984; Nestorowicz *et al.*,1985). It appears that peptides are unable to form the correct conformation for antibody recognition of the intact loop (Shapira *et al*,.1984).

These examples demonstrate that predicting regions of a protein which can be used as synthetic immunogens is difficult even with a three dimensional structural model. Thus it is speculative to attempt to predict the structure of the RRV E2 neutralization epitopes between amino acids 209 and 253 based on the failure of peptides to raise antibodies that reacted

with native RRV. As discussed above, it seems likely that the antigenic sites in this region are largely discontinuous but this does not exclude the possibility of antigenic loops formed by stretches of continuous sequence which are conformationally constrained and not mimicked by peptides.

Chapter 3

Examination of early events in cell entry of RRV using penetration and binding assays and the selection of further variants of RRV resistant to mAb T1E7

3.1 Introduction

Although alphaviruses are rapidly becoming understood at the genome level, the molecular nature of early events in alphavirus-cell interactions is poorly understood (see Chapter 1). Productive receptor molecules on the cell surface have not been identified and binding sites on the virion for such receptors have not been defined. The alphavirus surface proteins E1 and E2 must mediate cell attachment and entry. By defining sites involved in attachment and penetration on these proteins, it may be possible to develop a better understanding of cell surface receptors and their role in virus infection *in vitro*. This may identify virus determinants of tissue tropism and pathogenesis *in vivo*.

Because antibodies may neutralize alphaviruses by blocking attachment to cells (Roehrig *et al.*,1988; see Chapter 1) it is possible that some neutralization determinants on virus proteins are involved in receptor recognition or are close to receptor binding sites. Earlier studies with RRV had indicated that two mAb T1E7 escape mutants of RRV T48 had increased penetration rates in BHK cells compared to the parent virus (Vrati, 1986). The variants examined had amino acid changes in epitope b2 (Tv1, Asp246→Asn, and Tv3, Thr248→Pro). The triple variant Tv161 with changes at epitopes a, b1 and b2 (Thr216→Ile; Lys234→Asn; Asp246→Asn) also penetrated faster in BHK cells than T48 (Vrati, 1986). The penetration rates of mAb escape mutants changed solely at epitopes a or b1 has not been examined.

The penetration assay used by Vrati (1986) was from Baric *et al.* (1981). This assay measures the percent of virus able to initiate productive infection at 36°, as judged by plaque formation, after the addition of polyclonal antiserum at various times. Virus which has "penetrated" is resistant to neutralization and so forms plaques. Thus the percentage of

virus which has penetrated at a given time after the inoculum was added to the cell monolayers can be calculated by comparison with controls to which no antiserum was added (see Methods). Virus is presumably susceptible to neutralization by added antibody until it is actually internalized (see Chapter 1). Therefore the penetration assay does not differentiate between faster attachment leading to faster entry and acceleration of post-attachment events such as receptor recruitment, translocation to coated pits and endocytosis which could also result in faster entry. The penetration assay differs from cell binding assays in that it measures productive infection and uses very small amounts of virus, approximately 100 pfu.

In addition to increased rates of penetration, variants of T48 altered in epitope b2 were reported to have retarded growth, RNA synthesis and protein synthesis in BHK cells compared to T48 and epitope a or b1 variants (Vrati, 1986). This suggested that a single amino acid change in E2 might be affecting not only penetration of RRV but might also be slowing uncoating and release of the viral genome from the endosome into the cytoplasm.

This chapter describes attempts to test the hypothesis that changes in epitope b2 increase the penetration rate of RRV in tissue culture and to assess whether changes in the a and b1 epitopes, at residues 216 and 232-234 of E2, affect penetration. To define the extent of possible amino acid changes in the b2 epitope seven further T1E7 resistant variants were selected and the E2 gene sequenced in the region of the b2 epitope. By examining the penetration characteristics of a series of natural isolates and laboratory variants of RRV the role of epitope b2 in fast penetration was further defined. In preliminary experiments the binding of RRV T48 to cultured cells was also examined using radiolabelled virus.

3.2 Materials and Methods

3.2.1 Virus stocks

The stocks of T48 and NB5092, have been described in Chapter 2. NB0/10/7 is a mouse passage derived variant of NB5092 with only (Lys251→Asn) amino acid alteration in E2 and no changes in E1 (Meek *et al.*,1989). RRV dE2 is a variant of RRV T48 with a deletion from amino acid 55 to 61 in E2 (Dalgarno *et al.*,1983; Vrati *et al.*,1986)

Laboratory selected mAb resistant variants of T48 (Vrati *et al.*,1988) were described in part in Chapter 2. For clarity those mAb variants used in this chapter are described in Table 3.1. Geographic isolates of RRV used in penetration assays are described in Table 3.2.

Virus stocks were prepared as described in Chapter 2. To prepare independent clones of T48 for mAb T1E7 selection, plaques were picked from BHK cell monolayers and amplified in BHK cells to produce working stocks. Titration was on Vero and BHK monolayers as in Chapter 2.

3.2.2 Cell culture

The growth of Vero and BHK cells is described in Chapter 2. Mosquito cells (*Aedes albopictus*, C6/36) cells were grown in Eagle's basal medium (EBM) supplemented with 10% FCS, at 28°, 5% CO_2.

3.2.3 Plaque assays

Plaque assays were as described in Chapter 2

3.2.4 Plaque Reduction Neutralization Assays (PRNA)

These assays were as described in Chapter 2.

Table 3.1

Laboratory selected mAb resistant variants of RRV T48 used in this chapter[1]

Virus	Selecting mAb	Amino acid change and epitope[2]
Tv1	T1E7	Asp246→Asn (b2)
Tv2	T1E7	Arg251→Ser (b2)
Tv5	T1E7	Asp246→Val (b2)
Tv42	T10C9	Thr216→Ile (a)
Tv61	NB3C4	Lys234→Gln (b1)
Tv62	NB3C4	Lys234→Ile (b1)
Tv63	NB3C4	Lys234→Asn (b1)
Tv64	NB3C4	Lys234→Glu (b1)
Tv161	T10C9	Thr216→Ile (a)
	NB3C4	Lys234→Asn (b1)
	T1E7	Asp246→Asn (b2)

[1]Data shown is from Vrati *et al.* (1988).

[2]Amino acids are numbered from the N-terminus of the E2 glycoprotein of RRV T48; epitopes are shown in brackets.

Table 3.2

Geographic variants of RRV used in penetration assays

Isolate[1]	Area and year of isolation	Reference
PB629	Lake Poomah, Vic., 1974	Marshall *et al.* (1982)
GG2227	Gol Gol, NSW, 1974	Marshall *et al.* (1982)
BH38019	Barmah Forest, Vic., 1983	Dr. I.D. Marshall, (unpublished)
F9073	Fiji, 1979	Aaskov *et al.* (1981)

[1]All isolates were obtained from Dr. I.D. Marshall (John Curtin School of Medical Research, Canberra, Australia).

3.2.5 Antiserum

The production of high titre polyclonal antiserum and mAb T1E7 ascitic fluid is described in Chapter 2. Antiserum was incubated at 56° for 20 min prior to use.

3.2.6 Penetration assays

The method used for penetration assays was that described by Baric *et al.*,(1981) as adapted by Vrati (1986). Penetration was taken to be the percentage of virus which had acquired resistance to neutralization by added polyclonal antibodies compared to controls to which no antibody was added. The basic method was as follows: cell monolayers (BHK or Vero) in 60mm tissue culture dishes were washed twice with HBSS (pH 7.2) and inoculated with ~100 pfu of virus in 300µl of HBSS. Monolayers were incubated at 36°, with frequent rocking, for 10, 20, 40 or 60 min. At each time point the appropriate monolayers were removed from the incubator and the inoculum aspirated. Antiserum (0.5ml of 1/10 dilution of anti-RRV mouse ascitic fluid) was added and the monolayers incubated for a further 10 min. Antiserum was aspirated, the monolayers washed twice with HBSS and overlaid with agar for plaque development. Control monolayers were incubated for 60 min, the inoculum was aspirated, the monolayers washed twice with HBSS and overlaid. Monolayers were stained with neutral red 24-30 hours post-infection (pi). Assays were in triplicate and the mean at each time point was expressed as a percentage of the mean control value. For experiments at different pH values, HBSS was either prepared at pH 8.0 using HEPES buffer (Sigma, St Louis, MO, USA) or at pH 6.5 using PIPES buffer (Sigma).

Statistical analysis of penetration assays was designed and performed by Mr. Ross Cunningham, Dept. of Statistics, The Faculties,

Australian National University. It was assumed that the number of plaques had a Poisson distribution and that the means were related to time and virus in a multiplicative way. This led to a statistical model of the form: ln(expected number of plaques) = β_0+(effect due to time)+(time.virus interaction effect). This model is a special case of a powerful general class of statistical models known as "generalized linear models" (McCullagh and Neldes, 1990). Models of the above form were fitted to all sets of data analysed, the fits being extremely good. In all cases the interaction term was significant suggesting that the profiles differed between viruses.

Expected values together with standard errors were obtained from the fitted models. These summary data were appropriately backtransformed and are presented graphically in the text together with the original data shown as percent penetration.

3.2.7 Kinetics of viral RNA synthesis in BHK cells

For each virus eight BHK monolayers in 35mm tissue culture dishes were infected (moi~1). Control monolayers were mock infected with HBSS. After adsorbing for 60 min (36°), the inoculum was aspirated from each monolayer and the cells washed twice with HBSS; 2ml GMEM+10% BS was added and the monolayers returned to 36°. At appropriate times medium was aspirated and the monolayers washed twice with PBS (pH 7.2) and 0.5ml of EMEM containing AMD (5µg/ml) and [5-^{3}H] uridine (10µCi/ml) was added. Controls were incubated with and without AMD. After two hours incubation, the monolayers were washed twice with PBS and dissociated in 200µl of 1% sodium dodecyl sulphate (SDS; Bio-Rad Laboratories, Richmond, CA, USA). Duplicate 50µl samples were spotted onto glass-fibre discs, precipitated with 5% TCA and assayed in a Beckman liquid scintillation counter (Newton *et al.*,1981). For each time point, growth samples (100µl of culture fluid supernatant) were collected

into HBSS and assayed for extracellular virus (EV) by plaque assay on Vero cell monolayers.

3.2.8 Kinetics of virus growth in Vero cell monolayers

Vero cell monolayers in 60mm tissue culture dishes were infected (moi~1). EV titres were measured at appropriate times pi by plaque assay.

3.2.9 Labelling RRV with [^{35}S]-methionine

The method used for labelling virus was adapted from Raghow (1974). Confluent BHK monolayers in 60mm tissue culture dishes were infected with RRV T48 (moi ~1). Four hours pi, growth medium was aspirated and the monolayers washed twice with EMEM-minus-methionine (EMEM-M), 5ml of EMEM plus [^{35}S]-methionine (5μCi/ml; NEN Research Products, Boston, MA, USA) was added to each dish and the dishes incubated at 36°/5% CO_2 for a further 19 hours. Supernatants were harvested and 5ml of EMEM-M added to the monolayers which were incubated for a further 15 hours. Supernatants were clarified by centrifugation at 10K rpm for 10 min at 4°, virus was precipitated with one fourth volume polyethylene glycol (40% in NET) and purified on a sucrose gradient as described in Chapter 2. Fractions were collected and samples of each precipitated with 5% TCA and counted in a liquid scintillation counter to identify the peak fractions. Peak fractions were plaque assayed on Vero cell monolayers. The specific activity of virus labelled in this manner was 38 pfu/cpm in the peak fraction for the first harvest and 140 pfu/cpm in the second harvest. The peak of radiolabelled virus coincided with the peak infectivity fraction. Compared with the virus titres in growth samples taken prior to harvest an efficiency of 33% of infectious purified virus was obtained. Protein concentrations in purified virus preparations were estimated using the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA, USA). Particle:pfu ratios were estimated from the virus

titre and the protein concentration following the method of Marsh and Helenius (1980).

3.2.10 Virus binding assays

BHK cell monolayers in 35mm dishes were chilled to 4°washed twice with ice cold HBSS (pH 7.2) and an estimated 30,000 cpm of ^{35}S-labelled virus was added (pfu:cell~0.75) per monolayer and incubated at 4° for between 10 and 60 min. At the indicated time the inoculum was aspirated and the monolayers washed three times with ice cold HBSS. Following this 200µl of 1% Nonidet P-40 (Sigma) was added and the monolayers scraped into 10ml of scintillation cocktail (see Chapter 2) for counting. Mock infected monolayers were used as controls. In some experiments 1% SDS was used to disrupt cell monolayers and in one experiment samples were split, with one fraction TCA precipitated prior to counting and the other counted directly; no differences were noted between methods. Experiments were also performed at room temperature (approximately 20°) and at 37°.

3.2.11 Selection of mAb T1E7 resistant variants of RRV T48

120µl of T48 clones 1, 2 and 3 ($3x10^6$-$6x10^6$ pfu) were independently incubated with 120µl of mAb T1E7 ascitic fluid (diluted 1:10 in HBSS) for 60 min at 37°. This mixture was then further diluted 1/10 in HBSS and 100µl aliquots added to Vero monolayers; 20 monolayers per incubation mix were inoculated. Adsorption was for 60 min at 36° and monolayers were then overlaid for plaque development. Monolayers were stained with neutral red (0.1%) 36 hours post-infection and 12 well isolated plaques were selected from each incubation. Plaques were amplified in BHK monolayers and the supernatants were harvested when the monolayers showed cytopathic effect (cpe). Supernatants (10^{-4} dilution) were screened for resistance to mAb T1E7 in a PRNA using a 1/50 dilution of T1E7. Controls were incubated with normal ascitic fluid. T48 and Tv1 were screened in

parallel with these assays as positive and negative controls. Those clones with 50% or greater resistance to mAb T1E7 were titrated against mAb T1E7 in a PRNA. Resistant stocks were plaque purified on Vero monolayers, amplified in BHK cells and the resulting supernatants titrated against mAb T1E7 in a PRNA.

Virus was designated 1, 2 or 3 for the original clonal stock from which it was selected followed by a number from 1-12 representing the plaque picked following selection with T1E7. The entire number was prefaced with E7. Thus E7/1/3 represents the third plaque picked from clone 1 of T48 after selection with T1E7.

3.2.12 RNA extraction from infected cells

High molecular weight RNA for sequence analysis was extracted from BHK cells essentially using the method of Shine and Dalgarno (1973). Confluent BHK cell monolayers were infected with virus (moi 0.5-1). At 16-18 hours pi the growth medium was removed, the cells washed twice with PBS and nucleic acids extracted with 6% sodium p-aminosalicylate and phenol/m-cresol/8-hydroxyquinoline (4ml of each). This extract was transferred to 12ml disposable tubes (Sarstedt, West Germany) and held on ice while intermittently vortexing for 10 min. The phases were separated by centrifugation (10K rpm, 20 min, 4°). The aqueous phase was re-extracted with 0.5 volume of phenol / m-cresol / 8-hydroxyquinoline following the addition of 0.2 volumes of 15% (w/v) NaCl solution. Following centrifugation as before, nucleic acids in the aqueous phase were precipitated with 2.5 volumes of ethanol and dissolved in 0.45ml of water. 0.1 volumes of x10 NET (1.5M NaCl, 0.12M Tris (pH 7.4), 0.02M EDTA) was added followed by 0.5ml of 4M LiCl in water. The mixture was frozen in liquid nitrogen, thawed, and high molecular weight RNA pelleted by centrifugation, resuspended as above and reprecipitated with 4M LiCl.

RNA was resuspended in NET buffer and precipitated twice with ethanol; the final pellet was resuspended in 5mM Tris (pH 7.4), 0.01mM EDTA. The RNA concentration was measured spectrophotometrically, adjusted to 4- 5µg/µl for sequencing, aliquoted and stored at -70°.

3. 2.13 Dideoxynucleotide sequencing of viral RNA

Nucleotide sequencing followed the procedure of Faragher and Dalgarno (1986). Total intracellular RNA (IC/RNA) extracted from virus infected cells was used for all reactions.

Primer/template mix: 3.5µl of IC/RNA (16µg) was mixed with 1.0µl of primer (5-20pmol/µl) and incubated at 65-80° for 5 min, quick-cooled on wet ice and 1.0µl of 0.4M KCl added. 1.0µl of this mixture was added to each reaction tube. The primers used were E2 1987, GATGATGCGCTCAGA; E1 2752, TCCACCCCACATGAA; E1 3178, GTATGGCACATGCAC; E1 3386, ACCTGGCAGCTCAGG; E1 3556, CGCCGTGGAAAAGTG; (primers are listed 5' to 3'; the priming site is indicated; numbering is from the 5' end of the RRV NB5092 26S RNA, Faragher *et al.*, 1988).

Reaction mix: this consisted of 50mM Tris (pH 8.3), 8mM MgCl, 50mM KCl, 0.4mM dithiothreitol (DTT), 50µM of each of the deoxynucleoside triphosphates (dNTP) (dTTP, dATP, dGTP) with the exception of dCTP which was 2.5µM,and one of the dideoxynucleoside triphosphates (ddNTP); ddCTP (0.125 µM), ddTTP (5.0µM), ddATP (5.0µM), ddGTP (4.0µM). For some reactions 0.5x these ddNTP concentrations were used. To this was added 10µCi [α^{32}P]-dCTP, 4.0 U AMV reverse transcriptase (Molecular Genetics Resources, Florida, USA) and 1.0µl of primer / template to give a final volume of 5µl.

After incubation at 42.5° for 30 min. 1.0µl of chase (0.5mM of each of dCTP, dATP, dTTP, dGTP) was added to each reaction tube and the

reactions incubated for a further 20 min at 42.5°. Reactions were stopped by the addition of 10-20µl of sample buffer (96% deionized formamide, 25mM tris-borate (pH8.3), 0.5mM Na_2EDTA, xylene cyanol, bromophenol blue), followed by heating at 90° for 2 min. Reaction products were separated on 40cm long, 0.4mm thick, 6% polyacrylamide gels (acrylamide:bis acrylamide 19:1; Bio-Rad). Gels were exposed to Kodak RX medical X-ray film.

3.3 Results

3.3.1 Penetration rate of RRV T48 and mAb resistant variants of T48 in BHK cells

Previous work (Vrati, 1986) had demonstrated that variants of RRV T48 with amino acid changes in epitope b2 had increased penetration rates in BHK cells compared to T48. It was not known whether changes in epitopes a and b1 also altered penetration rates. To compare the penetration rates of T48 and mAb resistant variants altered in epitopes a, b1 and b2 in BHK cells, penetration assays were performed as described in the Methods (3.2.6). There was no difference in penetration kinetics between T48, the epitope a mutant (Tv42) or the epitope b1 mutant, (Tv61). However, the epitope b2 mutant (Tv1) had a greater proportion of virus resistant to neutralization, compared to T48, at every time point, as previously demonstrated by Vrati (1986). A statistical method for handling these data was developed (R. Cunningham; see Methods; 3.2.6) which allowed upper and lower confidence intervals to be plotted on a logarithmic transformation of the plaque numbers at each time point expressed as a proportion of the plaque numbers in the 60 minute controls. The resulting curves together with 95% confidence intervals are shown together with the results expressed as percentage penetration (Fig 3.1).

Figure 3.1

Penetration of mAb resistant variants of RRV T48 in BHK cells

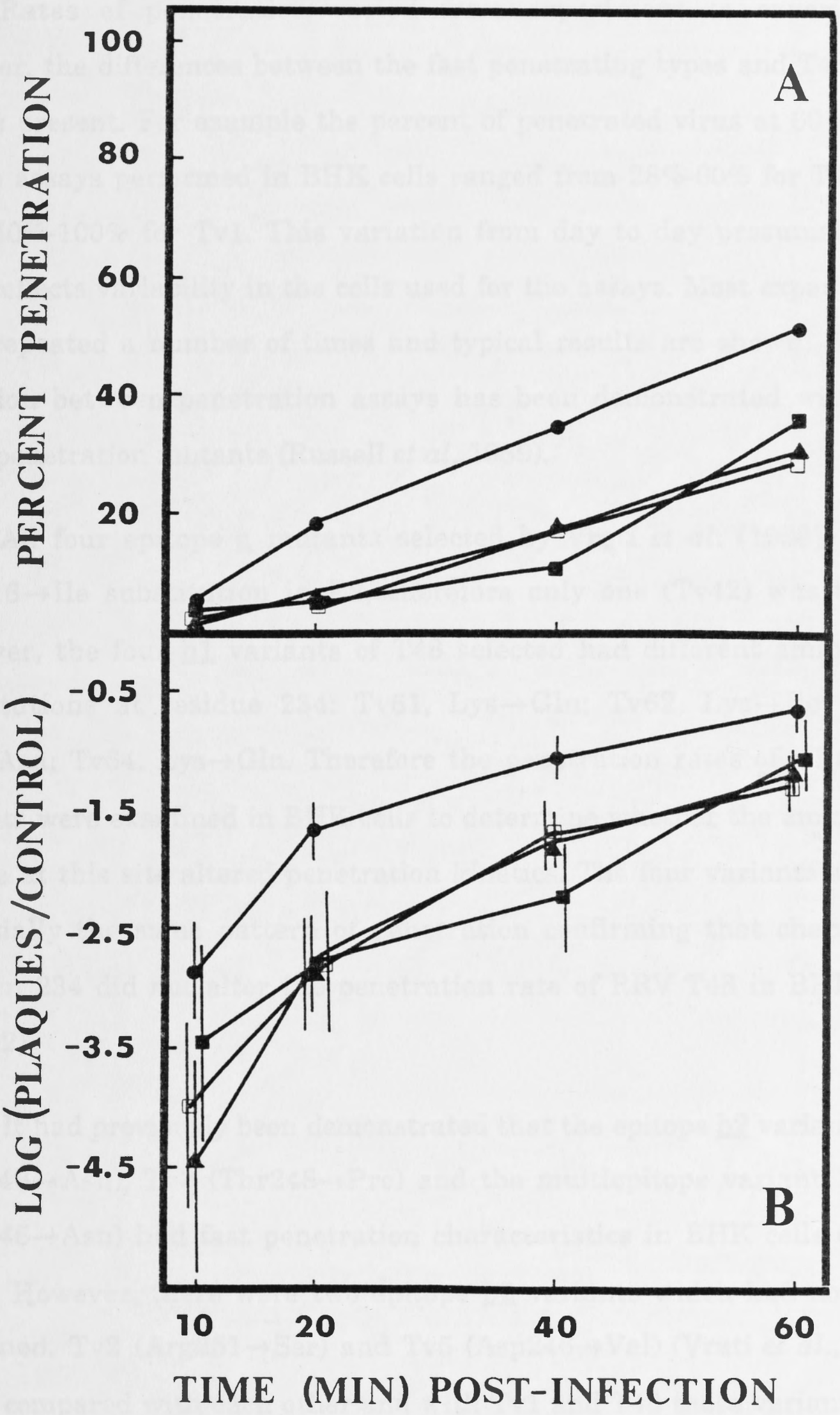
BHK cell monolayers in 60mm dishes were infected with ~100 pfu of RRV T48 or the mAb resistant variants, Tv1, Tv42, and Tv61 and incubated at 36°. At designated times the inoculum was removed and 0.5ml of polyclonal anti-RRV ascitic fluid (diluted 1:10 in HBSS, pH 7.2) was added. After a further 10 min the ascitic fluid was aspirated and the monolayers washed twice with HBSS (pH 7.2) and overlaid for plaque development. The number of plaques obtained after 60 min adsorption, HBSS wash but no antibody treatment was taken as 100%. Each assay was performed in triplicate and average values for each time point expressed as a percentage of the 60 minute controls (3.1A). This represents the proportion of virus resistant to neutralization at each time point. The same data is shown (3.1B) as a logarithm of the plaque numbers at each time point expressed as a proportion of the numbers in the 60 min controls. This allows statistical analysis of the data, as described in the Methods; 95% confidence limits are shown as error bars.

Tv1 ●

Tv42 ■

Tv61 □

T48 ▲


A
B
100
80
60
40
20
-0.5
-1.5
-2.5
-3.5
-4.5
10
20
40
60
PERCENT PENETRATION
LOG (PLAQUES /CONTROL)
TIME (MIN) POST-INFECTION

Rates of penetration varied from experiment to experiment, however, the differences between the fast penetrating types and T48 were always present. For example the percent of penetrated virus at 60 min in all the assays performed in BHK cells ranged from 28%-60% for T48 and from 50%-100% for Tv1. This variation from day to day presumably, in part, reflects variability in the cells used for the assays. Most experiments were repeated a number of times and typical results are shown. Similar variation between penetration assays has been demonstrated with SIN rapid penetration mutants (Russell *et al.*,1989).

All four epitope a mutants selected by Vrati *et al.* (1988), had a Thr216→Ile substitution in E2, therefore only one (Tv42) was tested. However, the four b1 variants of T48 selected had different amino acid substitutions at residue 234: Tv61, Lys→Gln; Tv62, Lys→Ile; Tv63, Lys→Asn; Tv64, Lys→Glu. Therefore the penetration rates of all the b1 variants were examined in BHK cells to determine whether the amino acid change at this site altered penetration kinetics. The four variants showed essentially the same pattern of penetration confirming that changes at position 234 did not alter the penetration rate of RRV T48 in BHK cells (Fig 3.2).

It had previously been demonstrated that the epitope b2 variants Tv1 (Asp246→Asn), Tv3 (Thr248→Pro) and the multiepitope variant, Tv161 (Asp246→Asn) had fast penetration characteristics in BHK cells (Vrati, 1986). However, there were two epitope b2 variants which had not been examined, Tv2 (Arg251→Ser) and Tv5 (Asp246→Val) (Vrati *et al.*,1988). When compared with each other and with Tv1 and T48 these variants also had fast penetration rates (Fig 3.3). Tv5 consistently had somewhat faster early penetration compared to the other viruses assayed. For further work, Tv1 was chosen as a representative of the epitope b2 mutants for two

Figure 3.2

Penetration of epitope b1 variants of RRV T48 in BHK cells

Percentage penetration in BHK cells (3.2A) and statistical analysis of the same data (3.2B) of the epitope b2 variants Tv61, Tv62, Tv63 and Tv64 are shown. Assays were performed in triplicate exactly as described in Figure 3.1. Error bars represent 95% confidence limits.

Tv61 ▲

Tv62 ■

Tv63 ●

Tv64 □

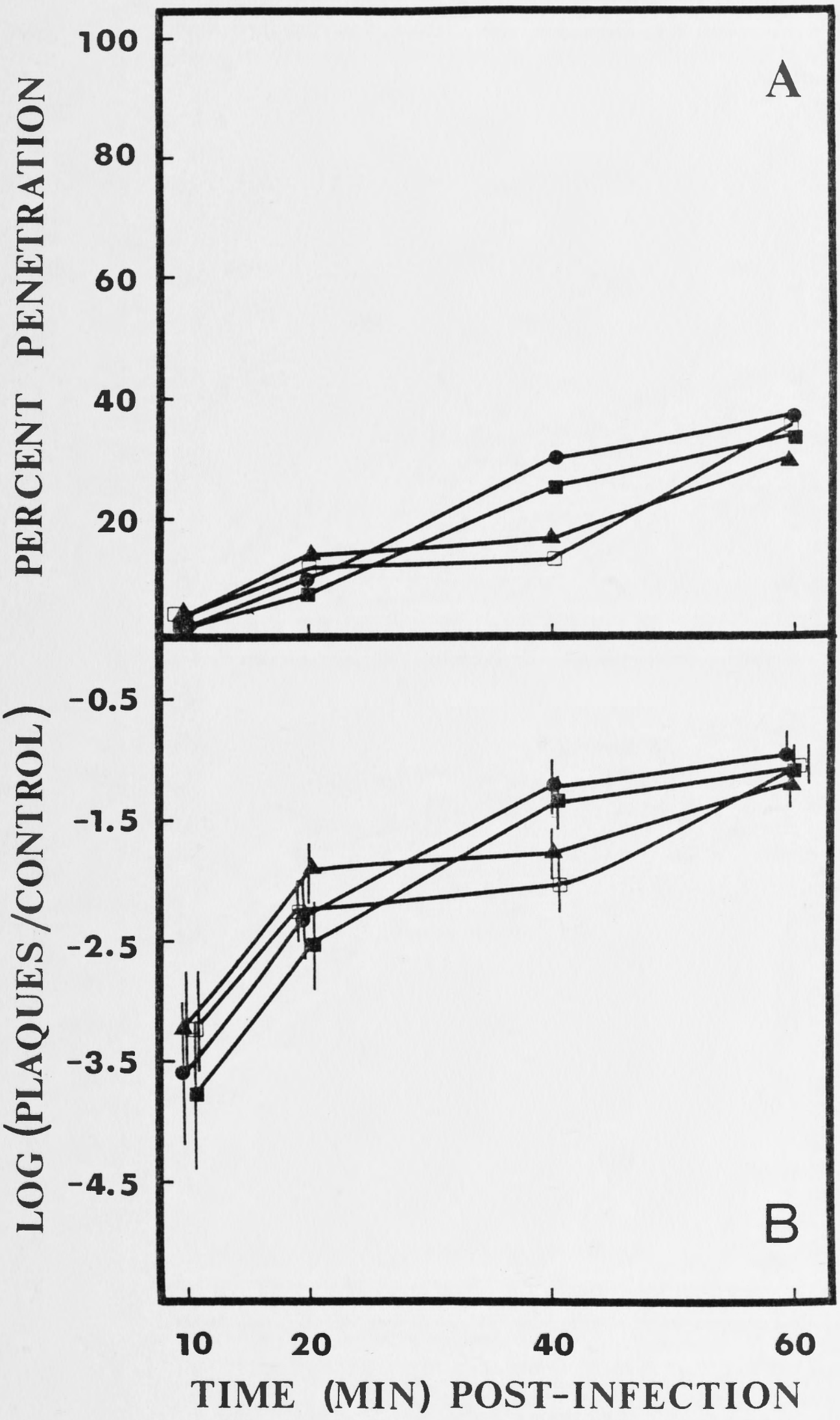

PERCENT PENETRATION
100
80
60
40
20
A
LOG (PLAQUES /CONTROL)
-0.5
-1.5
-2.5
-3.5
-4.5
B
10
20
40
60
TIME (MIN) POST-INFECTION

Figure 3.3

Penetration of epitope b2 variants of RRV T48 in BHK cells

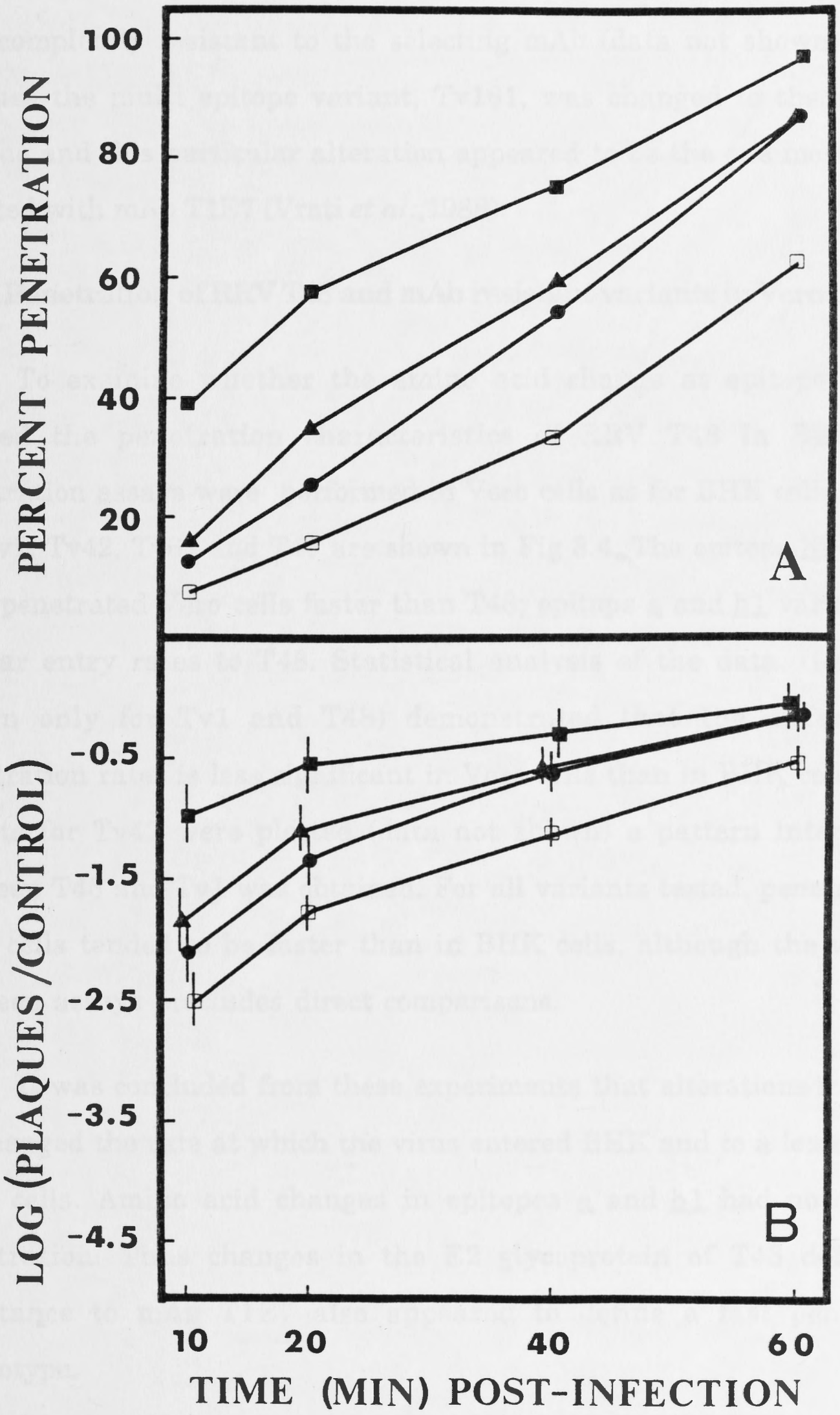
Percentage penetration in BHK cells (3.3A) and statistical analysis of the same data (3.3B) of the epitope b2 variants Tv1, Tv2, Tv5 and of the parental T48 are shown. Assays were in triplicate as described in Fig 3.1. Error bars represent 95% confidence limits.

Tv1 ●

Tv2 ▲

Tv5 ■

T48 □


PERCENT PENETRATION
100
80
60
40
20
A
LOG (PLAQUES /CONTROL)
-0.5
-1.5
-2.5
-3.5
-4.5
B
10
20
40
60
TIME (MIN) POST-INFECTION

reasons: (1) because it was the only representative of these variants that was completely resistant to the selecting mAb (data not shown) and (2) because the multi epitope variant, Tv161, was changed to the same b2 residue and this particular alteration appeared to be the one most readily selected with mAb T1E7 (Vrati *et al.*,1988).

3.3.2 Penetration of RRV T48 and mAb resistant variants in Vero cells

To examine whether the amino acid change at epitope b2 only altered the penetration characteristics of RRV T48 in BHK cells, penetration assays were performed in Vero cells as for BHK cells. Results for Tv1, Tv42, Tv61 and T48 are shown in Fig 3.4. The epitope b2 variant, Tv1, penetrated Vero cells faster than T48; epitope a and b1 variants had similar entry rates to T48. Statistical analysis of the data, (for clarity shown only for Tv1 and T48) demonstrated that the difference in penetration rates is less significant in Vero cells than in BHK cells. When results for Tv42 were plotted (data not shown) a pattern intermediate between T48 and Tv1 was obtained. For all variants tested, penetration in Vero cells tended to be faster than in BHK cells, although the variation between assays precludes direct comparisons.

It was concluded from these experiments that alterations in epitope b2 changed the rate at which the virus entered BHK and to a lesser extent Vero cells. Amino acid changes in epitopes a and b1 had no effect on penetration. Thus changes in the E2 glycoprotein of T48 defined by resistance to mAb T1E7 also appeared to define a fast penetration phenotype.

Figure 3.4

Penetration of RRV T48 and mAb resistant variants in Vero cells

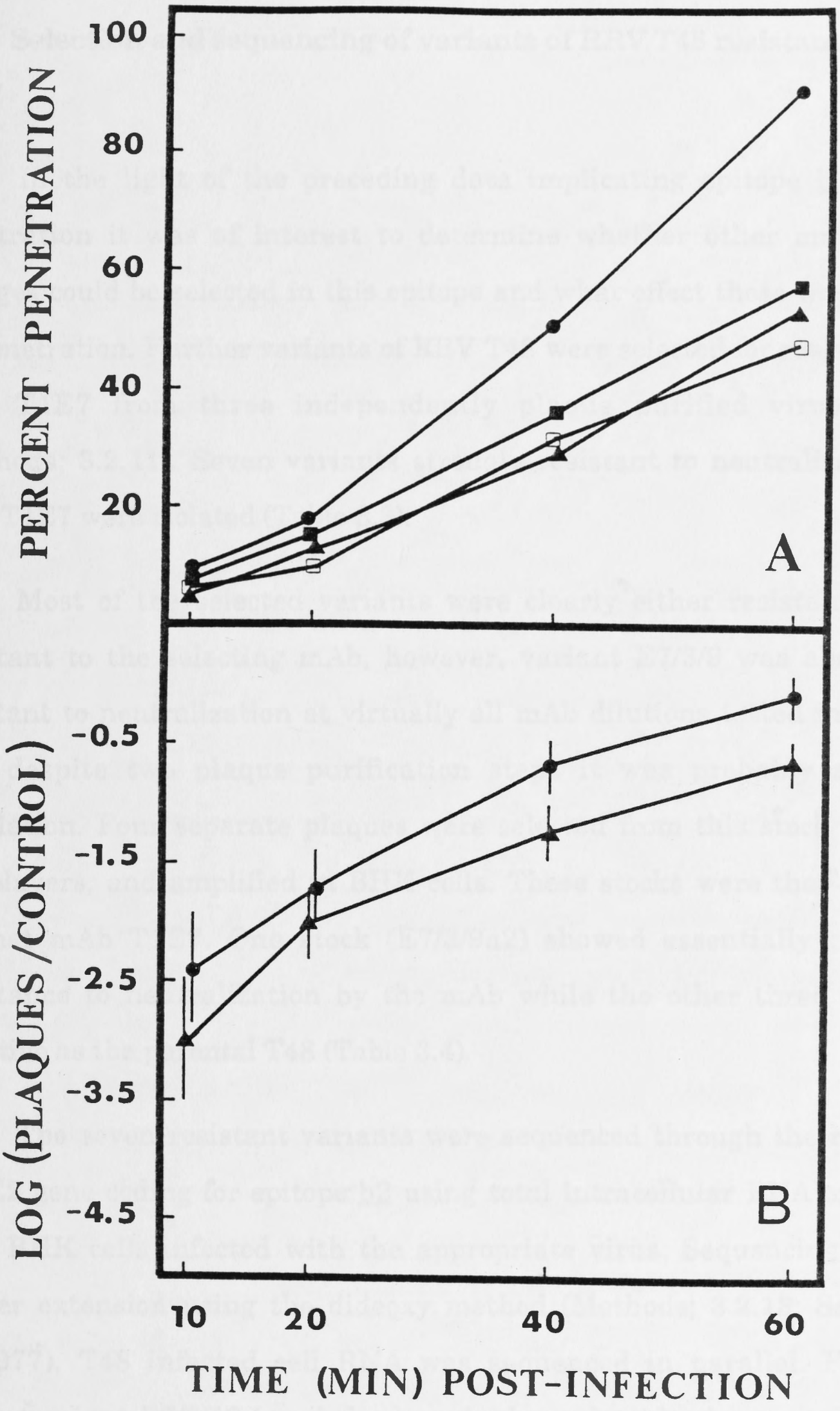
Penetration assays were performed on Vero cell monolayers exactly as for BHK cell monolayers (Fig 3.1). The percent penetration of T48, Tv1, Tv42 and Tv61 is shown (3.4A). Statistical analysis of the data is presented (3.4B) for T48 and Tv1 only. Error bars represent 95% confidence limits.

Tv1 ●

Tv42 ■

Tv61 □

T48 ▲


PERCENT PENETRATION
100
80
60
40
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A
LOG (PLAQUES /CONTROL)
-0.5
-1.5
-2.5
-3.5
-4.5
B
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60
TIME (MIN) POST-INFECTION

3.3.3 Selection and sequencing of variants of RRV T48 resistant to mAb T1E7

In the light of the preceding data implicating epitope b2 in cell penetration it was of interest to determine whether other amino acid changes could be selected in this epitope and what effect these would have on penetration. Further variants of RRV T48 were selected for resistance to mAb T1E7 from three independently plaque purified virus stocks (Methods; 3.2.11). Seven variants strongly resistant to neutralization by mAb T1E7 were isolated (Table 3.3).

Most of the selected variants were clearly either resistant or not resistant to the selecting mAb, however, variant E7/3/9 was about 50% resistant to neutralization at virtually all mAb dilutions tested indicating that despite two plaque purification steps it was probably a mixed population. Four separate plaques were selected from this stock on Vero monolayers, and amplified in BHK cells. These stocks were then titrated against mAb T1E7. One stock (E7/3/9a2) showed essentially complete resistance to neutralization by the mAb while the other three were as sensitive as the parental T48 (Table 3.4).

The seven resistant variants were sequenced through the region of the E2 gene coding for epitope b2 using total intracellular RNA extracted from BHK cells infected with the appropriate virus. Sequencing was by primer extension using the dideoxy method (Methods; 3.2.13; Sanger *et al.*,1977), T48 infected cell RNA was sequenced in parallel. For each variant except E7/3/12 (see below), a single nucleotide change was found. This change coded for a nonconservative amino acid alteration in the b2 epitope (Table 3.5). Four of the six variants sequenced had the Asp246→Asn alteration which produced a new potential N-linked glycosylation site at residue 246. The other two changes found were

Table 3.3

Neutralization of mAb T1E7 selected variants of RRV T48 by mAb T1E7

Selected variant	Neutralization titre[1]
E7/1/1	<50
E7/1/8	100
E7/1/12	<50
E7/2/8	100
E7/3/6	200
E7/3/9	50*
E7/3/12	200
T48	400,000

[1] Titres are the reciprocals of the lowest antibody dilution at which greater than or equal to 50% neutralization occurred; two-fold dilutions were used starting from 1:50.

* 3/9 appears to be a mixed population with approximately 50% of the virus neutralized at all dilutions tested (see text).

Table 3.4

Neutralization[1] by mAb T1E7 of plaque purified stocks selected from RRV E7/3/9

	Antibody Dilution[2]						
Virus	**50**	**100**	**200**	**400**	**800**	**1600**	**3200**
T48	100	100	100	98	94	92	94
3/9	47	51	43	39	49	49	60
3/9a1	100	100	100	97	97	98	95
3/9a2	10	23	9	0	23	18	21
3/9a3	100	100	100	99	97	99	94
3/9a4	100	100	100	98	92	92	93

[1]The percentage neutralization at each antibody dilution, in a PRNA, for the parental T48 and the mAb selected variant E7/3/9 and four plaque purified stocks derived from E7/3/9, shown as 3/9a1 to 3/9a4.

[2]Antibody dilutions are shown as the reciprocal of the dilution of mAb T1E7 ascitic fluid used.

Table 3.5

Sequence differences in E2 of mAb T1E7 selected variants of RRV[1]

RRV T48 variant	[2]Position of nucleotide change	Codon change	Amino acid change[3]
E7/1/1	1786	GAU→AAU	Asp246→Asn
E7/1/8	1803	AGG→AGU	Arg251→Ser
E7/1/12	1786	GAU→AAU	Asp246→Asn
E7/2/8	1786	GAU→AAU	Asp246→Asn
E7/3/6	1802	AGG→ACG	Arg251→Thr
E7/3/9a2	1786	GAU→AAU	Asp246→Asn
E7/3/12	nd	nd	nd

[1]Nucleotide and amino acid differences from the parental RRV T48 strain are shown. [2]Nucleotides are numbered fom the 5' end of the T48 26S RNA (Dalgarno *et al.*,1983). [3]Amino acids are numbered from the N terminus of E2.

nd not determined (see text).

Arg251→Ser and Arg251→Thr. Only the Arg251→Thr change was not seen by Vrati *et al.* (1988) and this is unlikely to result in different properties to the Arg251→Ser mutation which was found by Vrati *et al.* (1988). The Asp246→Asn alteration was found in all three selection series. In each case this resulted from a G→A nucleotide transversion which might be expected to occur more frequently than the other two changes found, both of which require transitions. For E7/3/12 a four nucleotide compression between nucleotides 1,786-1,789 prevented determination of the sequence at this point. This compression covers the amino acid 246 codon and is not found in T48, therefore it is likely that a nucleotide alteration has occurred in this codon in E7/3/12 possibly corresponding to the Asp246→Val mutation seen by Vrati *et al.* (1988). These workers also found a change at amino acid 248 (Thr→Pro); no changes at this position were found in the current study.

3.3.4 Penetration of RRV NB5092 in BHK and Vero cells

RRV NB5092 is a natural isolate of RRV that has undergone much less laboratory adaptation than RRV T48. The complete sequence of the two strains of RRV has been determined (Faragher *et al.*,1988). There are five amino acid differences in E2 and three in E1 between these isolates; these are the only proteins that are likely to be involved in cell penetration. NB5092 is approximately 100 times more resistant to neutralization by mAb T1E7 than is T48 (Vrati *et al.*,1988) and this may be due to a conservative amino acid difference (Arg251→Lys) between NB5092 and T48 E2. NB5092 cannot be differentiated from T48 in neutralization assays with polyclonal anti-T48 sera (Chapter 2) or with mAbs T10C9 and NB3C4 (C. Fernon and R. C. Weir, unpublished) and therefore provided a test of whether alteration from the T48 sequence in the b2 epitope affected penetration rate in an isolate of RRV somewhat closer in its passage history to the field situation.

The results of a penetration experiment in BHK cells using NB5092 and T48 are shown in Fig 3.5. In this experiment Tv161 was used as a fast entry control. Also included is NB0/10/7, a variant of NB5092 derived by mouse passaging, which has a single, non-conservative Lys251→Asn alteration in E2 (Meek *et al.*,1989). Both NB5092 and NB0/10/7 had the fast penetration phenotype compared to T48 with 90-100% of virus being resistant to neutralization by 60 min compared to 36% for T48. Similar differences in penetration for NB5092 and T48 were obtained in Vero cells with approximately 83% and 53% respectively being resistant to neutralization by 60 min (Fig 3.6).

These results appear to confirm that changes from the RRV T48 sequence in the T1E7 epitope lead to a fast penetration phenotype and suggest that the effect was not charge dependent since Arg 251→Lys, Lys 251→Asn, Asp 246→Val, Thr 248→Pro or Asp 246→Asn all gave similar penetration phenotypes. However, NB5092 does have other amino acid changes from T48 in E1 and E2 (Faragher *et al.*, 1988). The effect of these changes on the rate of penetration could not be defined.

3.3.5 Penetration of geographic variants of RRV in BHK cells

To further test the premise that epitope b2 was a determinant of penetration rate of RRV in tissue culture, four more geographic variants of the virus were examined. There was no data on the penetration of geographic variants of RRV other than NB5092 and T48. However, the E2 sequence for a number of isolates of RRV had been determined (Vrati, 1986; Burness *et al.*, 1988). To define penetration characteristics of RRV more completely four of these variants were chosen (Table 3.2). Based on the reported sequence these had differences to T48 in the b2 epitope (F9073 : Arg251→Lys; Burness *et al.*, 1988), near the b2 epitope (GG2227

Figure 3.5

Penetration of RRV T48, NB5092, NB0/10/7 and Tv161 in BHK cells

Percentage penetration in BHK cells (3.5A) and statistical analysis of the same data (3.5B) of the RRV variants NB5092, the mouse passage derived NB0/10/7 and the triple mAb resistant variant Tv161 are shown compared with T48. Assays were performed in triplicate as described in Fig 3.1. Error bars represent 95% confidence intervals.

NB5092 ●

NB0/10/7 ■

Tv161 □

T48 ▲

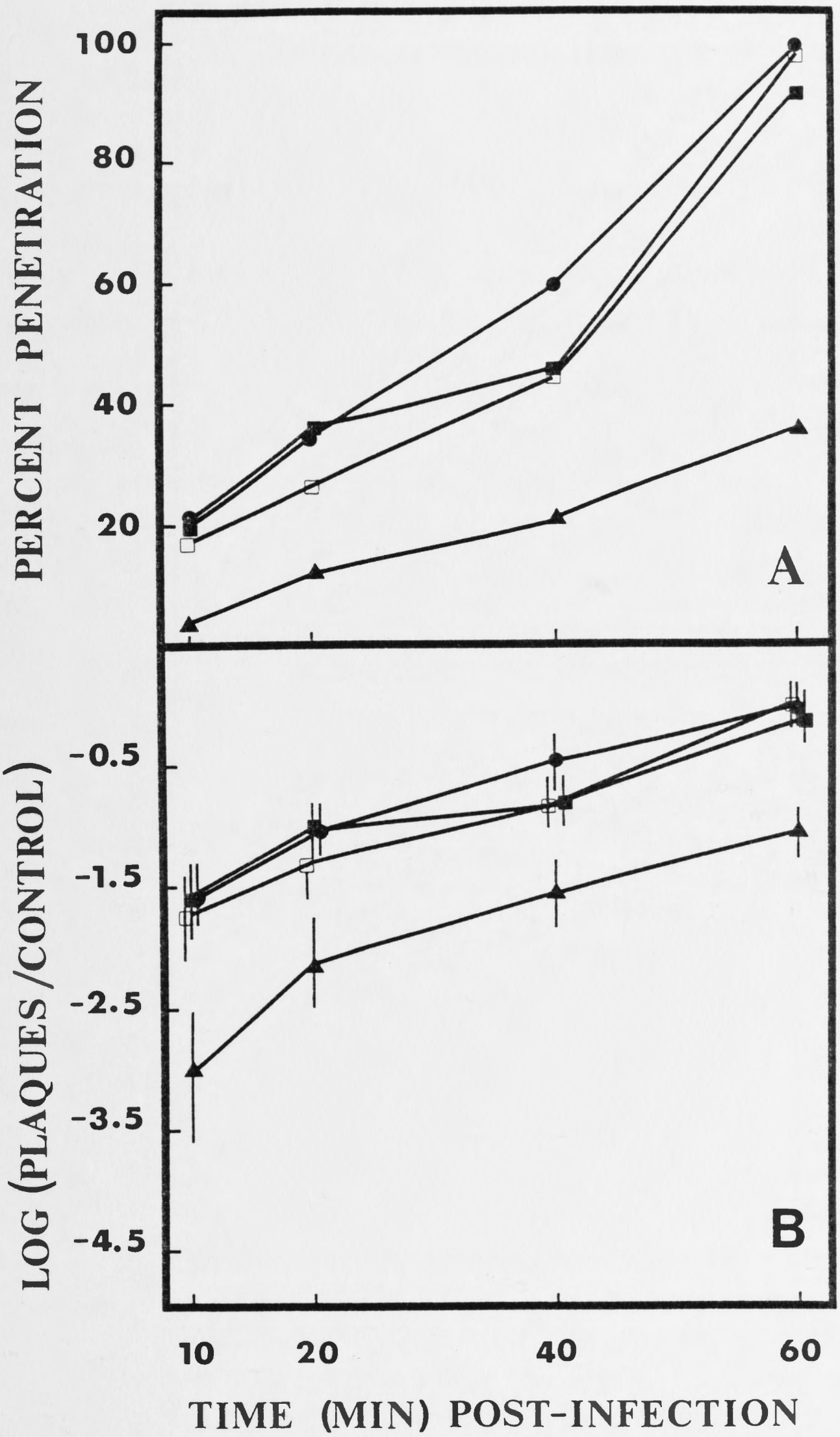

PERCENT PENETRATION
100
80
60
40
20
A
LOG (PLAQUES /CONTROL)
-0.5
-1.5
-2.5
-3.5
-4.5
B
10
20
40
60
TIME (MIN) POST-INFECTION

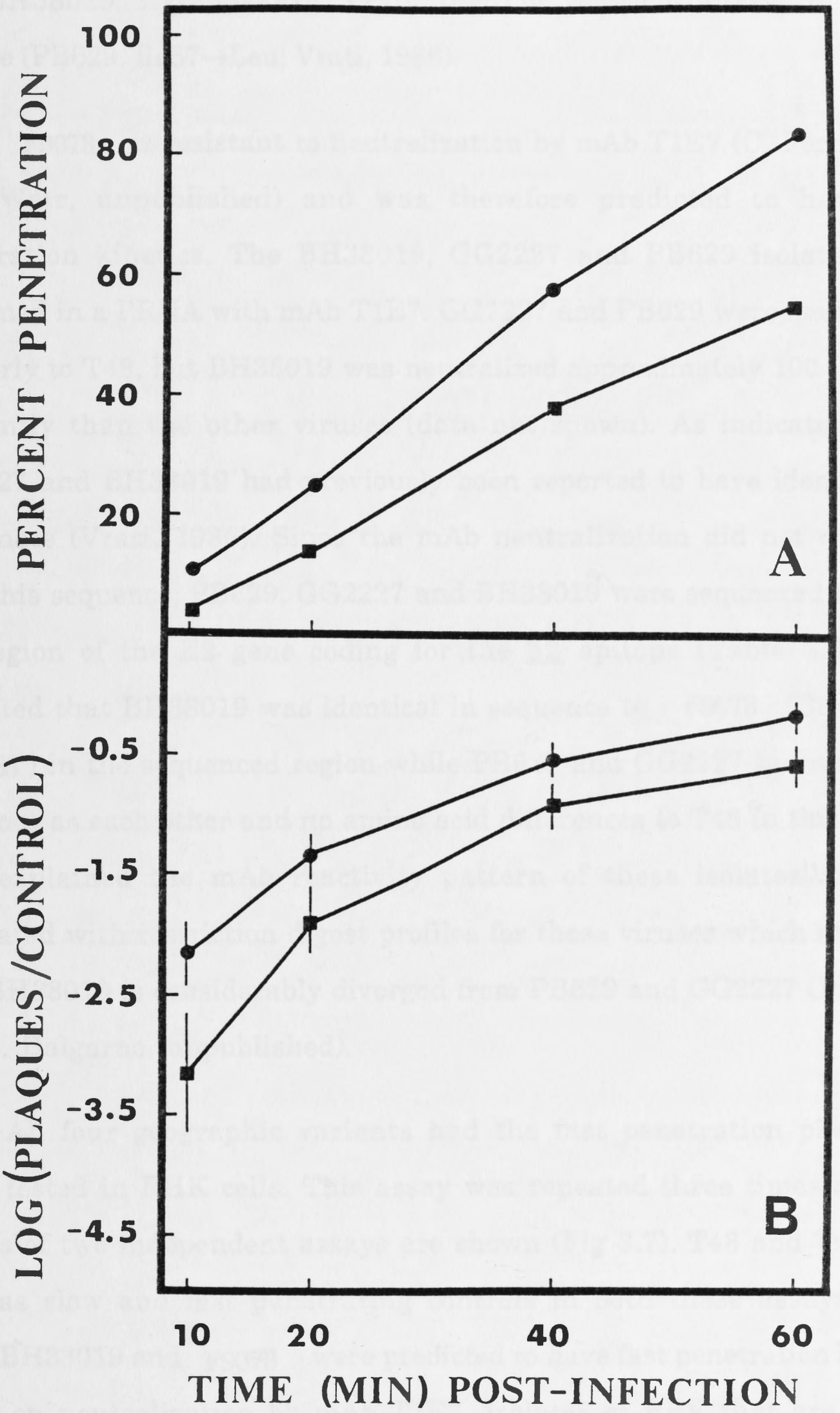
Figure 3.6

Penetration of RRV NB5092 and T48 in Vero cells

Percentage penetration in Vero cells (3.6A) and statistical transformation of the same data (3.6B) of RRV T48 and NB5092 are shown. Assays were in triplicate as described in Fig 3.1. Error bars represent 95% confidence limits.

NB5092 ●

T48 ■


PERCENT PENETRATION
100
80
60
40
20
A
LOG (PLAQUES /CONTROL)
-0.5
-1.5
-2.5
-3.5
-4.5
B
10
20
40
60
TIME (MIN) POST-INFECTION

and BH38019: Lys253→Arg; Vrati, 1986) or were not altered in the b2 epitope (PB629: Ile67→Leu; Vrati, 1986).

F9073 is resistant to neutralization by mAb T1E7 (C. Fernon and R.C. Weir, unpublished) and was therefore predicted to have fast penetration kinetics. The BH38019, GG2227 and PB629 isolates were examined in a PRNA with mAb T1E7. GG2227 and PB629 were neutralized similarly to T48, but BH38019 was neutralized approximately 100 fold less efficiently than the other viruses (data not shown). As indicated above GG2227 and BH38019 had previously been reported to have identical E2 sequences (Vrati, 1986). Since the mAb neutralization did not correlate with this sequence, PB629, GG2227 and BH38019 were sequenced through the region of the E2 gene coding for the b2 epitope (Table 3.6). This indicated that BH38019 was identical in sequence to F9073 (Burness *et al.*, 1988) in the sequenced region while PB629 and GG2227 had the same sequence as each other and no amino acid differences to T48 in this region. This explained the mAb reactivity pattern of these isolates and also correlated with restriction digest profiles for these viruses which indicated that BH38019 is considerably diverged from PB629 and GG2227 (P.J. Kerr and L. Dalgarno, unpublished).

All four geographic variants had the fast penetration phenotype when tested in BHK cells. This assay was repeated three times and the results of two independent assays are shown (Fig 3.7). T48 and Tv1 were used as slow and fast penetrating controls in both these assays. Thus while BH38019 and F9073 were predicted to have fast penetration kinetics based on neutralization by mAb T1E7, isolates of RRV that are closely related to T48 in their E2 amino acid sequence (PB629, GG2227) and are not altered in b2 also had the fast penetration phenotype. In summary, changes from the T48 sequence at epitope b2 increased penetration rate, however, natural isolates of RRV which are not altered in this epitope also

Table 3.6

Amino acid sequence differences in the E2 protein between RRV T48 and geographic variants[1]

	Virus					
	T48	**PB629**	**BH38019**	**GG2227**	F9073	**NB5092**
Amino acid						
3	Thr					Ile
67	Ile	Leu	Leu	Leu	Leu	Met
119	Asp					Asn
132	Asp				Asn	
203	Cys				Trp	
251	Arg		Lys		Lys	Lys
302	Leu					Val

[1]The amino acid differences from the T48 E2 protein for the geographic isolates of RRV used in this study are shown. A blank space indicates no change from T48. The T48 sequence is from Dalgarno *et al.* (1983); F9073 from Burness *et al.* (1988); NB5092 from Faragher *et al.* (1988); PB629, BH38019 and GG2227 are from Vrati (1986) and from sequence studies reported in this chapter (see text).

Figure 3.7

Penetration of geographic variants of RRV in BHK cells

Penetration assays were performed for the geographic variants of RRV: GG2227, PB627, BH38019 and F9073 in BHK cells. T48 and Tv1 were assayed in parallel with the geographic isolates. All assays were in triplicate as described in Fig 3.1. Data from two independent experiments (3.7A and 3.7B) are shown as the logarithm of the plaque numbers at each time point expressed as a proportion of the plaque numbers in the 60 minute controls. Error bars represent 95% confidence limits.

Tv1 ●

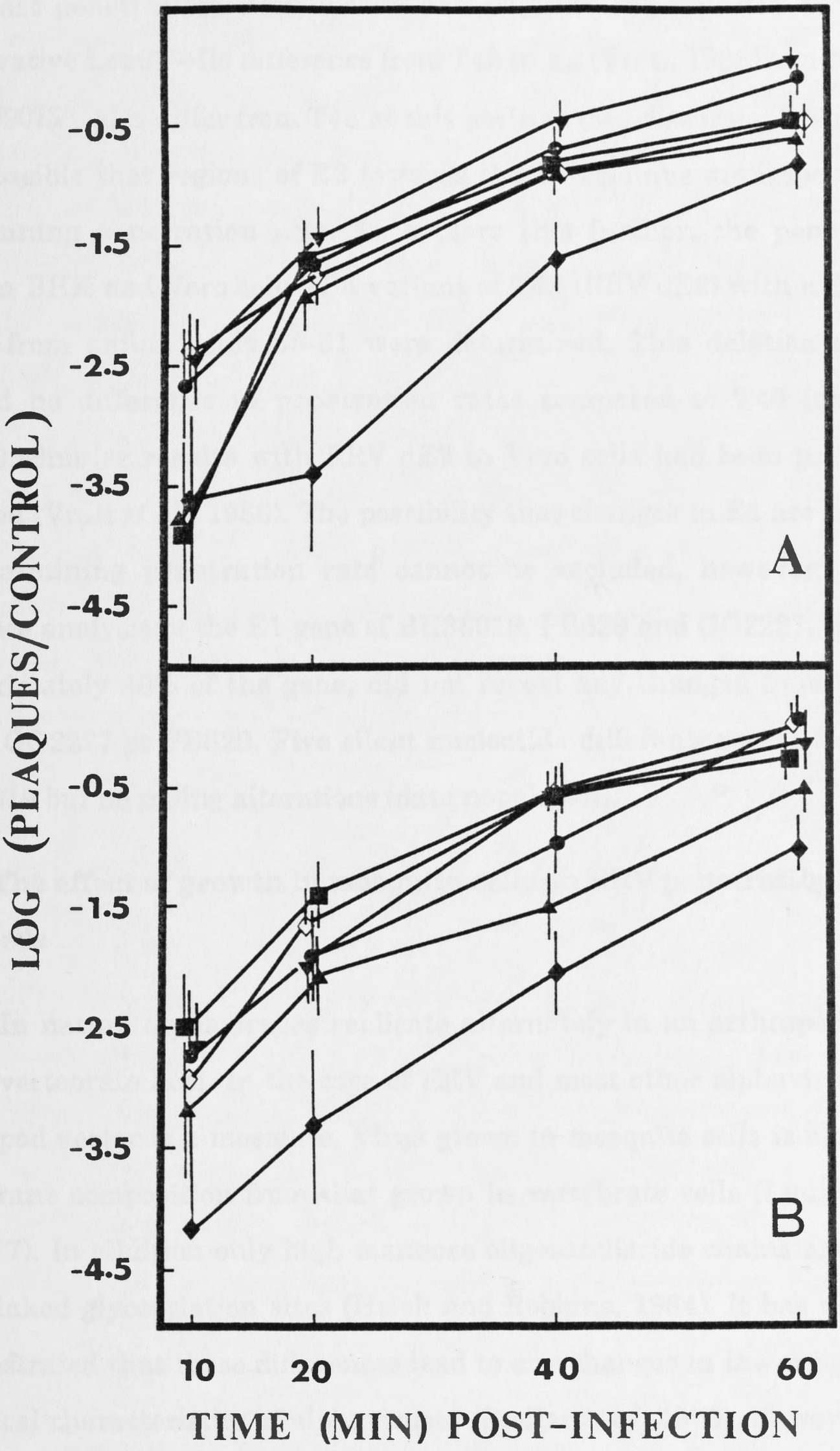
T48 ◆

F9073 ▲

BH38019 ▼

GG2227 ■

PB629 ◇


LOG (PLAQUES/CONTROL)
-0.5
-1.5
-2.5
-3.5
-4.5
A
B
10
20
40
60
TIME (MIN) POST-INFECTION

have fast penetration. Both PB629 and GG2227 are reported to have a conservative Leu67→Ile difference from T48 in E2 (Vrati, 1986) and NB5092 and F9073 also differ from T48 at this position (see discussion). Therefore it is possible that regions of E2 towards the N-terminus are important in determining penetration rate. To explore this further, the penetration rates in BHK and Vero cells of a variant of T48 (RRV dE2) with a deletion in E2 from amino acids 55-61 were determined. This deletion mutant showed no difference in penetration rates compared to T48 (data not shown). Similar results with RRV dE2 in Vero cells had been previously reported (Vrati *et al.*, 1986). The possibility that changes in E1 are involved in determining penetration rate cannot be excluded, however, partial sequence analysis of the E1 gene of BH38019, PB629 and GG2227, covering approximately 40% of the gene, did not reveal any changes from T48 for either GG2227 or PB629. Five silent nucleotide differences were found for BH38019 but no coding alterations (data not shown).

3.3.6 The effect of growth in mosquito cells on RRV penetration rate in BHK cells

In nature alphaviruses replicate alternately in an arthropod vector and a vertebrate host. In the case of RRV and most other alphaviruses the arthropod vector is a mosquito. Virus grown in mosquito cells is altered in membrane composition from that grown in vertebrate cells (Luukonen *et al.*,1977). In addition only high mannose oligosaccharide chains are added at N-linked glycosylation sites (Hsieh and Robbins, 1984). It has not been demonstrated that these differences lead to any changes in the antigenic or biological characteristics of alphaviruses (Stollar *et al.*,1976). However, it is possible that changes in the size or charge of the carbohydrate moieties on E1 and E2 could alter the interaction of the virion with the cell surface.

To examine the effect on cell penetration of host derived modifications of RRV, working stocks of RRV T48 or Tv1 were prepared in mosquito (C6/36) cells derived from *Aedes albopictus*. For both Tv1 and T48 the virus grown in mosquito cells penetrated BHK cells faster than virus grown in BHK cells and assayed in the same experiment. Figure 3.8 shows that 60% of T48(BHK) penetrated within 60 min while 76% of T48(mosq) penetrated in the same time. For Tv1 the corresponding values were 89 and 100%. Statistically these differences were very small (see Fig 3.8B), however, the same general pattern was observed in two experiments with Tv1 and three with T48. Assuming no genetic changes occurred during growth in mosquito cells, these results indicated that host derived phenotypic differences, such as the absence of complex oligosaccharide moieties may be important in cell penetration .

3.3.7 The effect of pH on penetration of RRV T48, Tv1 and NB5092

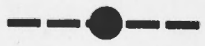
Evidence from the preceding section suggested that virus grown in mosquito cells and therefore lacking complex carbohydrate moieties penetrated faster than virus grown in BHK cells. It is possible that the absence of negatively charged sialic acid on the virion surface proteins enhanced penetration rate. This may be a direct effect of the reduction of negative surface charge on the virion. In support of this, the surface charge on the virion was an important determinant of the ability of SIN to infect mouse plasmacytoma cells (Symington and Schlesinger, 1978).

By altering the pH at which penetration assays were performed the surface charge on the cells and virions could be changed. In the following experiments the effect of three pH values (6.5, 7.2 and 8.0) on penetration rates in BHK cells was examined for Tv1, T48 and NB5092. All previous experiments used medium and HBSS at pH 7.2. For T48, penetration rate was virtually identical at all pH values (Fig 3.9). NB5092 had similar

Figure 3.8

Penetration in BHK cells of RRV T48 and Tv1 grown in either BHK or mosquito cells

RRV T48 and Tv1 were grown in either mosquito cells (C6/36) or BHK cells to prepare working stocks. Penetration assays in BHK cells were performed in parallel using virus prepared in each cell type. The percentage penetration with time for each virus is shown (3.8A) together with the statistical transformation of the same data (3.8B). All assays were performed in triplicate as described in Fig 3.1. Error bars represent 95% confidence intervals.

Tv1 (mosq) 

Tv1 (BHK) 

T48 (mosq) 

T48 (BHK) 

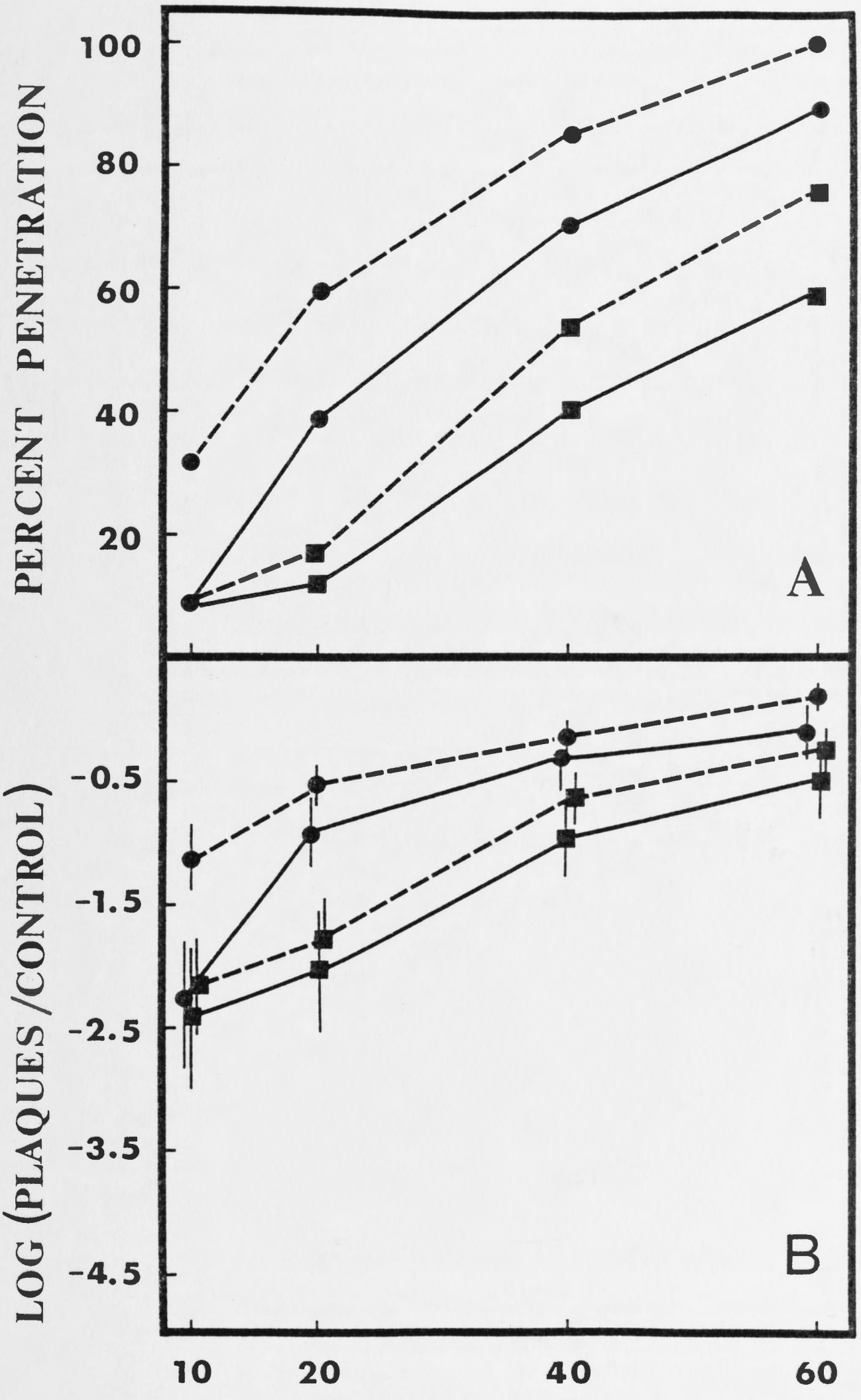
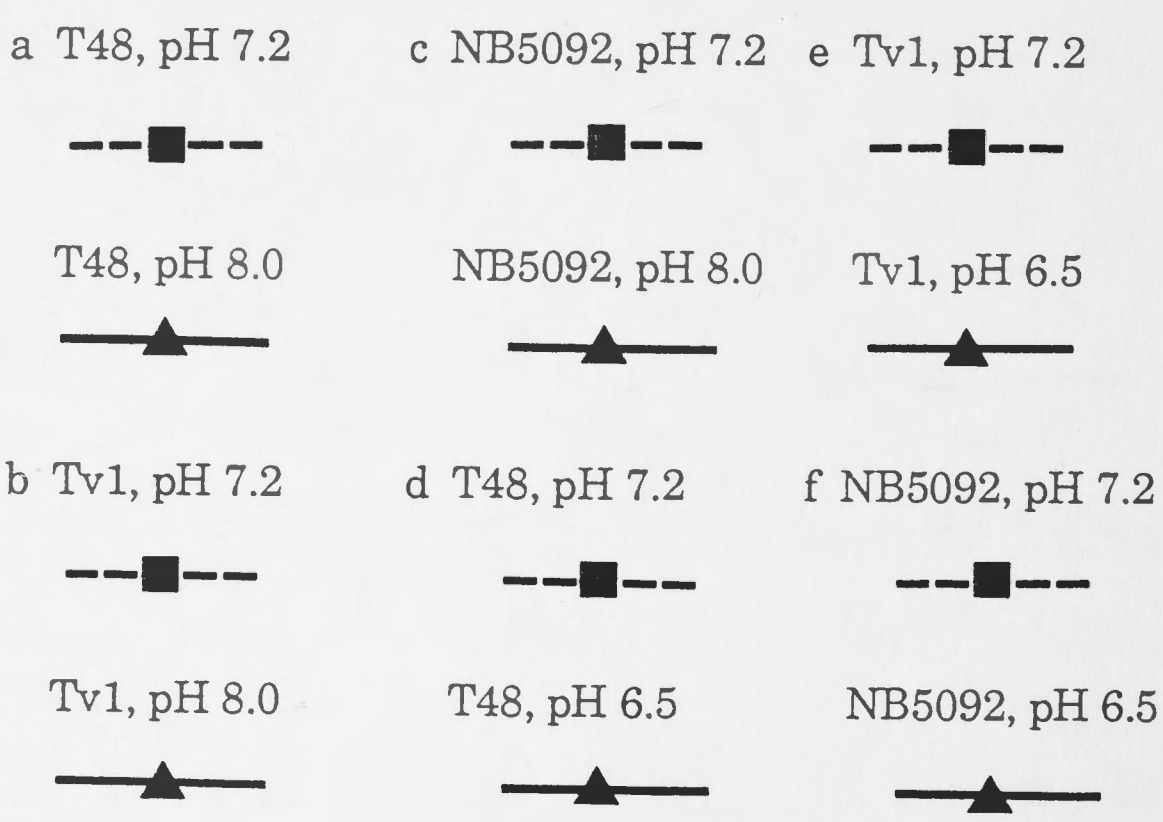
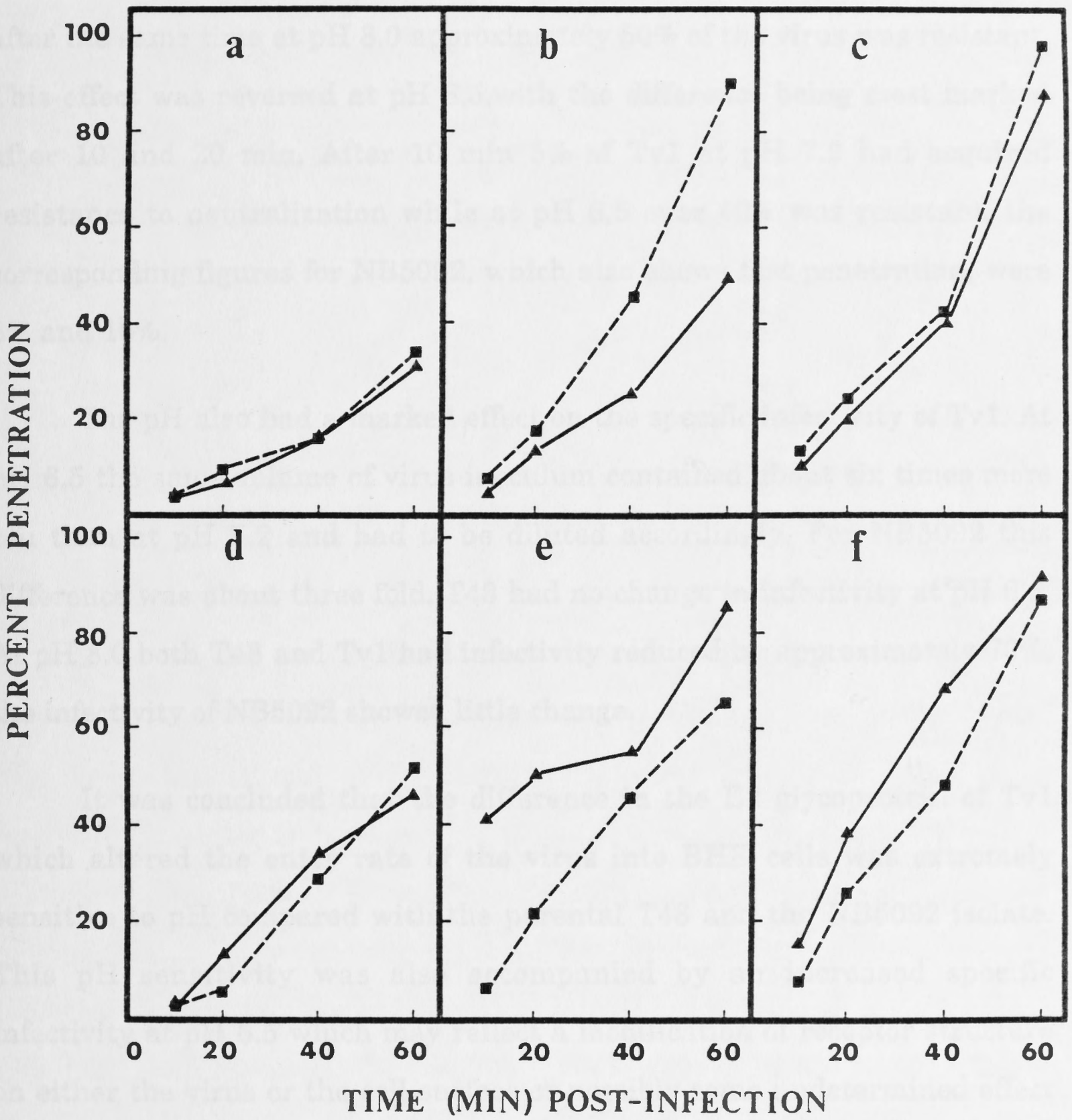

PERCENT PENETRATION
100
80
60
40
20
A
LOG (PLAQUES /CONTROL)
-0.5
-1.5
-2.5
-3.5
-4.5
B
10
20
40
60
TIME (MIN) POST-INFECTION

Figure 3.9

Penetration in BHK cells of RRV Tv1, T48 and NB5092 at pH 6.5, 7.2 and 8.0

BHK cell monolayers were washed twice with HBSS at the appropriate pH prior to infection with ~100 pfu of virus diluted in HBSS at the same pH. Incubation was at 36°. At designated times the inoculum was removed and 0.5ml of polyclonal anti-RRV ascitic fluid (diluted, 1:10 in HBSS of the appropriate pH) was added. After a further 10 min the ascitic fluid was aspirated and the monolayers washed twice with HBSS at the appropriate pH and overlaid for plaque development. The number of plaques obtained after 60 min adsorption, HBSS wash but no antibody treatment, was taken as 100%. Each assay was performed in triplicate and average values for each time point expressed as a percentage of the 60 minute controls. This represents the proportion of virus resistant to neutralization at each point. Assays on each graph were performed in parallel.




a
b
c
d
e
f
100
80
60
40
20
0
20
40
60
PERCENT PENETRATION
TIME (MIN) POST-INFECTION

penetration kinetics at pH 7.2 and 8.0, but was slightly accelerated at pH 6.5. Tv1 had markedly slower penetration at pH 8.0. For example at pH 7.2 at 60 min approximately 90% of Tv1 was resistant to neutralization while after the same time at pH 8.0 approximately 50% of the virus was resistant. This effect was reversed at pH 6.5,with the difference being most marked after 10 and 20 min. After 10 min 5% of Tv1 at pH 7.2 had acquired resistance to neutralization while at pH 6.5 over 40% was resistant, the corresponding figures for NB5092, which also shows fast penetration, were 8% and 16%.

The pH also had a marked effect on the specific infectivity of Tv1. At pH 6.5 the same volume of virus inoculum contained about six times more pfu than at pH 7.2 and had to be diluted accordingly. For NB5092 this difference was about three fold. T48 had no change in infectivity at pH 6.5. At pH 8.0 both T48 and Tv1 had infectivity reduced by approximately 70%; the infectivity of NB5092 showed little change.

It was concluded that the difference in the E2 glycoprotein of Tv1 which altered the entry rate of the virus into BHK cells was extremely sensitive to pH compared with the parental T48 and the NB5092 isolate. This pH sensitivity was also accompanied by an increased specific infectivity at pH 6.5 which may reflect a modification of receptor structure on either the virus or the cell surface or possibly some undetermined effect on virus aggregation. It is also possible that direct fusion of the Tv1 viral membrane with the plasma membrane of the cell may occur at this pH if the amino acid alteration in E2 alters the pH at which the fusion domain is exposed on the glycoprotein spike.

3.3.8 The effect of washing and salt concentration on penetration of RRV in BHK cells

The penetration assay used here measures the proportion of the virus inoculum which is resistant to neutralization by added antiserum at a particular time post-infection. It had not been determined if virus bound to cells but not penetrated was still susceptible to neutralization at all time points or whether only free virus was neutralized and therefore the assay was effectively measuring rates of virus attachment to cells.

To address this point the penetration rates of T48 and Tv1 were compared in BHK cells using the standard assay. In parallel a separate pair of assays was performed in which, instead of adding antibody at each time point, the cell monolayers were washed three times with HBSS pH 7.2 and then overlaid with agar for plaque development. By comparing the percent penetration after each time interval for washed or antibody treated monolayers an estimate of the proportion of virus firmly bound to cells, as judged by resistance to washing, but still susceptible to neutralization could be made. Because of the 60 min endpoint both viruses have 100% penetration in the wash only treatment at 60 min. The results (Fig 3.10) indicated that virus attached to cells was still susceptible to neutralization by added antibody at all time points. This suggested that the penetration assay was measuring a combination of rate of virus attachment to cells and rate of internalization of attached virus. The results showed that Tv1 was adsorbed to cells faster than T48 so a significant component of fast penetration appeared to be due to rapid attachment.

Two types of binding to cells have been postulated for SIN (Pierce *et al.*,1974): a weak binding where 40-50% of virus can be removed from the cells using a high salt (0.2M NaCl) wash and a tight binding where virus could not be washed from the monolayer. To examine whether such a

Figure 3.10

Penetration of RRV T48 and Tv1 in BHK cells without using antibody as a stop reagent

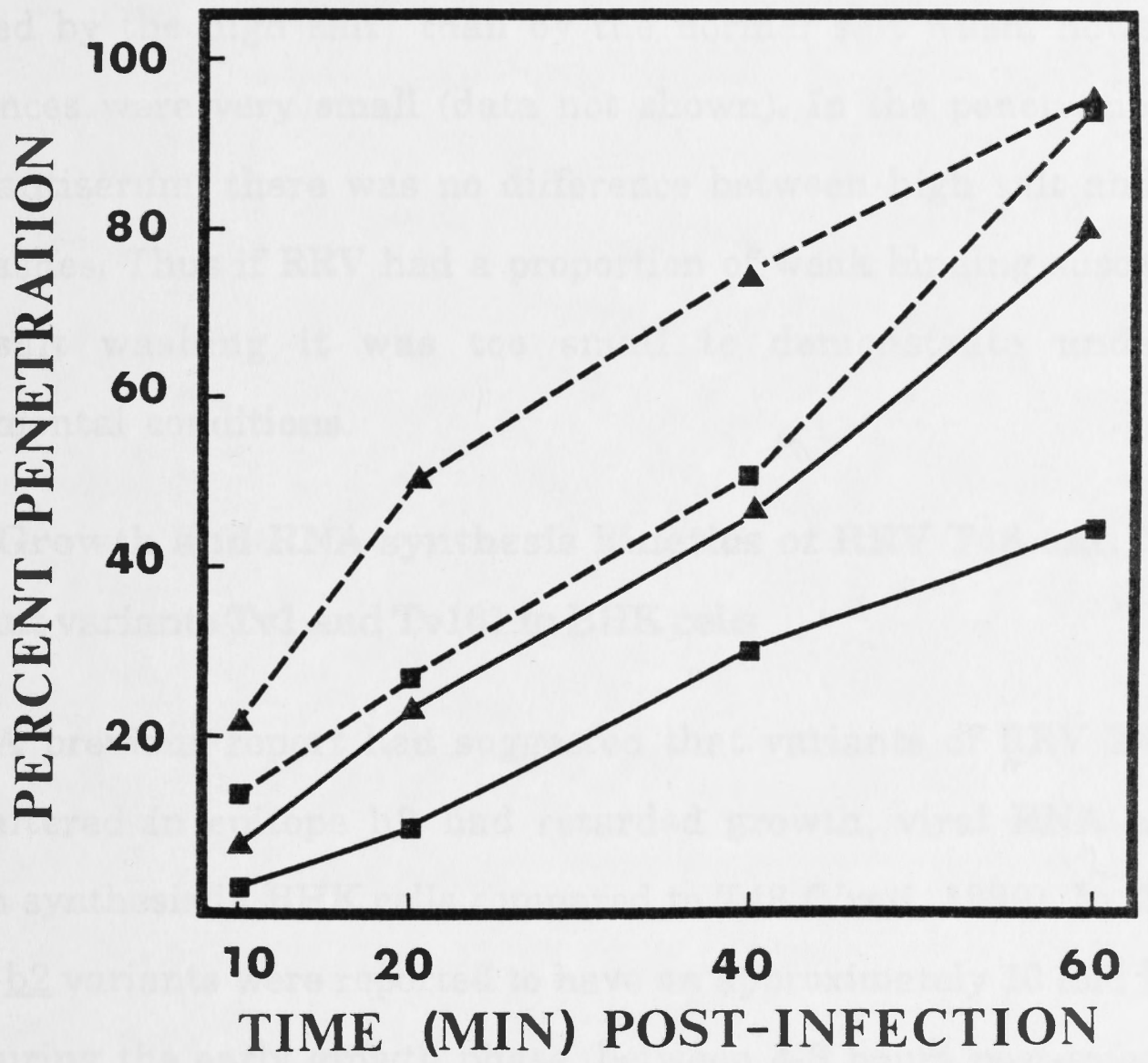
BHK cell monolayers in 60mm dishes were infected with~100 pfu of RRV T48 or the mAb resistant variant Tv1 and incubated at 36°. At designated times the inoculum was removed and the monolayers were either washed twice with HBSS (pH7.2) and overlaid for plaque development or 0.5ml of polyclonal anti-RRV ascitic fluid was added. After 10 min incubation the ascitic fluid was removed and the monolayers washed twice with HBSS and overlaid for plaque development. The number of plaques obtained after 60 min adsorption, HBSS wash but no antibody treatment, was taken as 100%. Each assay was performed in triplicate and average values for each time point were expressed as a percentage of the 60 min controls.

Tv1 (wash / no antibody)

Tv1 (antibody)

T48 (wash / no antibody)

T48 (antibody)


PERCENT PENETRATION
100
80
60
40
20
10
20
40
60
TIME (MIN) POST-INFECTION

weak binding occurred with RRV a penetration assay was done in which virus (T48) was either washed from the monolayer after each time interval or neutralized by the addition of antibody. Each treatment was performed in parallel with either a normal salt wash (HBSS) or a high salt wash (HBSS, 0.2M NaCl). In the wash experiments slightly more virus was removed by the high salt than by the normal salt wash, however, the differences were very small (data not shown). In the penetration assay, using antiserum, there was no difference between high salt and normal salt washes. Thus if RRV had a proportion of weak binding susceptible to high salt washing it was too small to demonstrate under these experimental conditions.

3.3.9 Growth and RNA synthesis kinetics of RRV T48 and the mAb resistant variants Tv1 and Tv161 in BHK cells

A previous report had suggested that variants of RRV T48 which were altered in epitope b2 had retarded growth, viral RNA and viral protein synthesis in BHK cells compared to T48 (Vrati, 1986). In that work all the b2 variants were reported to have an approximately 10 fold lower EV titre during the early growth phase, between 4-8 hours post-infection. No difference was shown with epitope a or b1 variants.

To re-examine these observations, BHK cell monolayers were infected with T48, the epitope b2 variant, Tv1 and the multiepitope variant Tv161 and virus growth and AMD resistant RNA synthesis determined. Growth curves are shown in Figure 3.11a. There was no difference in virus titres between these variants in the early stages of virus replication, up to 11 hours post-infection, although Tv1 reached somewhat higher final titres than T48 and Tv161. Similarly there was no appreciable difference in the time of onset of RNA synthesis for T48, Tv1 or Tv161 infected cells. However, the rates of RNA synthesis by Tv1 infected cells were higher

Figure 3.11

Kinetics of RRV T48, Tv1 and Tv161 growth and AMD resistant RNA synthesis in BHK cells

BHK cells were infected with RRV T48 or the mAb resistant variants Tv1 and Tv161 (moi~1) or mock infected (MI). Growth samples were collected from the culture mediums at the indicated times post-infection. Extracellular virus (EV) titres were determined by plaque assay on Vero cell monolayers (Fig 3.11a). At appropriate intervals monolayers from each infection were pulsed with EMEM containing 10μCi/ml [5-^{3}H]-uridine and 5μg/ml AMD. After incubation (2 hours, 36°), the medium was aspirated, monolayers washed twice with PBS (pH 7.2) and dissociated in 1% SDS. Acid precipitable radioactivity of duplicate 50μl aliquots was determined by liquid scintillation counting. Times indicated in the figure (3.11b) are the mid-points of the labelling periods. Control monolayers were mock infected with or without AMD.

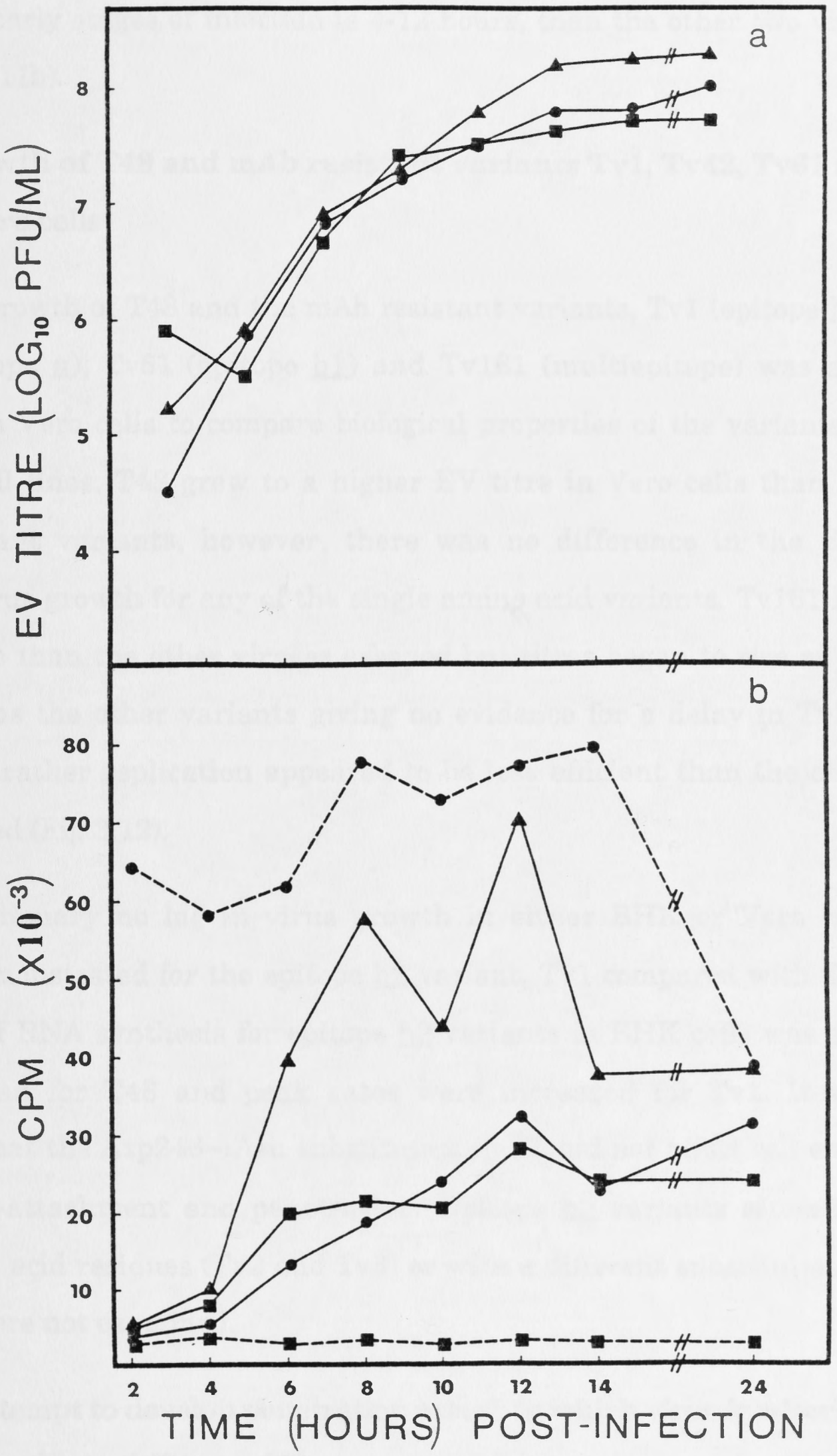
MI (no AMD)

MI (AMD)

Tv1

Tv161

T48


a
b
EV TITRE (LOG10 PFU/ML)
8
7
6
5
4
CPM (X10-3)
80
70
60
50
40
30
20
10
2
4
6
8
10
12
14
24
TIME (HOURS) POST-INFECTION

during the early stages of infection ie 4-12 hours, than the other two virus types (Fig 3.11b).

3.3.10 Growth of T48 and mAb resistant variants Tv1, Tv42, Tv61 and Tv161 in Vero cells

The growth of T48 and the mAb resistant variants, Tv1 (epitope b2), Tv42, (epitope a), Tv61 (epitope b1) and Tv161 (multiepitope) was also examined in Vero cells to compare biological properties of the variants in different cell lines. T48 grew to a higher EV titre in Vero cells than the mAb resistant variants, however, there was no difference in the time course of virus growth for any of the single amino acid variants. Tv161 had a lower titre than the other viruses assayed but titres began to rise at the same time as the other variants giving no evidence for a delay in Tv161 replication, rather replication appeared to be less efficient than the other viruses tested (Fig 3.12).

In summary no lag in virus growth in either BHK or Vero cells could be demonstrated for the epitope b2 variant, Tv1 compared with T48. The onset of RNA synthesis for epitope b2 variants in BHK cells was also no later than for T48 and peak rates were increased for Tv1. It was concluded that the Asp246→Asn substitution in E2 did not affect cell entry events post-attachment and penetration. Epitope b2 variants altered at other amino acid residues (Tv2 and Tv3) or with a different substitution at 246 (Tv5) were not examined.

3.3.11 An attempt to develop penetration assays in which virus is adsorbed to cells at 4° prior to shifting to 37°

An attempt was made to develop a penetration assay in which RRV was first adsorbed to cell monolayers at 4°, at which temperature little penetration will occur (Fries and Helenius, 1979), followed by washing to

Figure 3.12

Growth of mAb resistant variants of RRV in Vero cells

Vero cell monolayers were infected with RRV T48 or the mAb resistant variants Tv1, Tv42, Tv61 or Tv161 (moi~1). Growth samples were collected from the culture medium at the indicated times post-infection. Extracellular virus titres were determined by plaque assay on Vero cell monolayers.

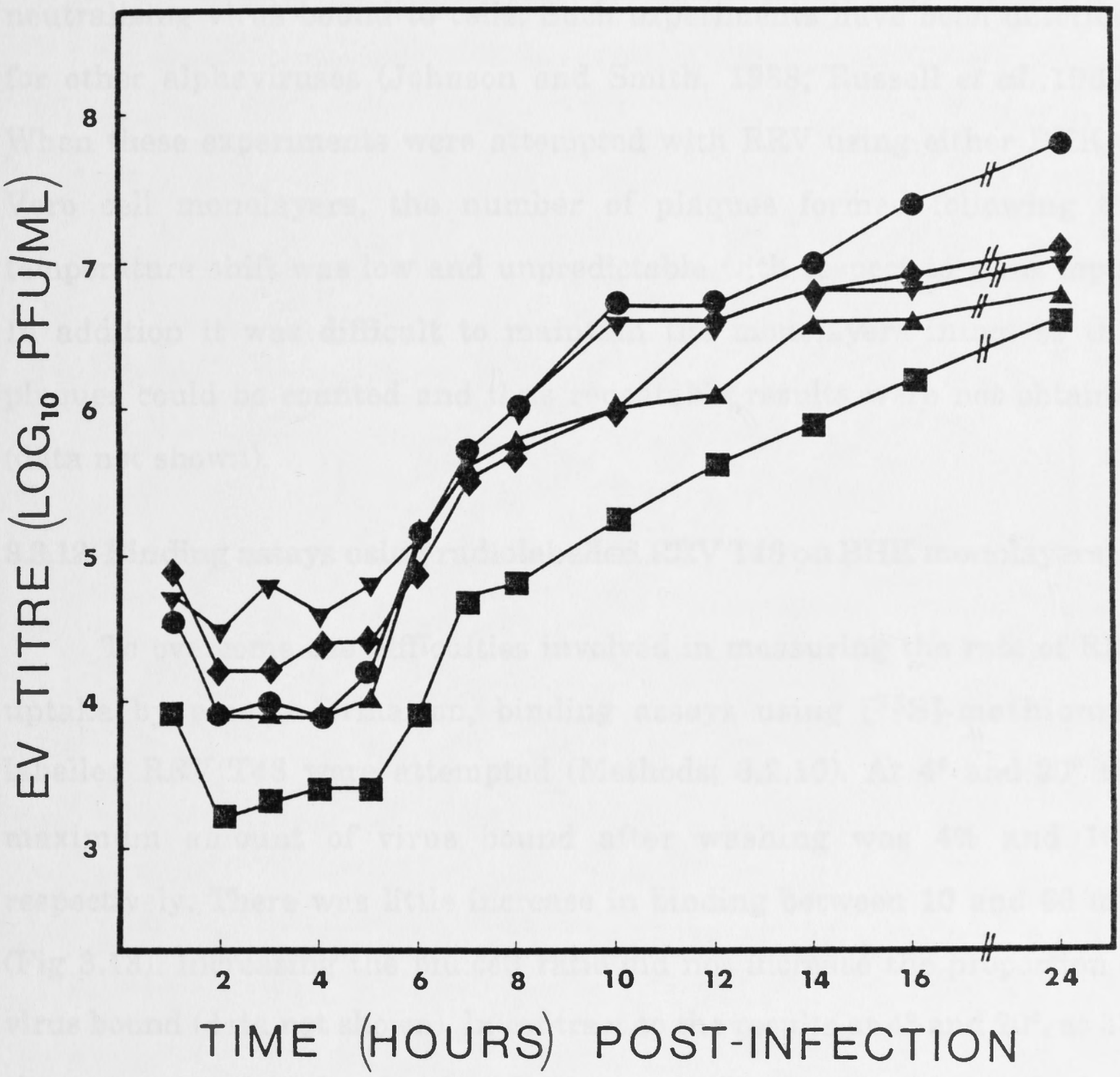
Tv1 ▲

Tv42 ◆

Tv61 ▼

Tv161 ■

T48 ●


EV TITRE (LOG_{10} PFU/ML)
8
7
6
5
4
3
2
4
6
8
10
12
14
16
24
TIME (HOURS) POST-INFECTION

remove unbound virus and a shift to 36° to allow penetration. In this way it was planned to separate differences in rates of cell attachment and cell entry. This would also provide a good system to examine whether the RRV neutralizing mAbs act by blocking virus attachment to cells or neutralizing virus bound to cells. Such experiments have been described for other alphaviruses (Johnson and Smith, 1988; Russell *et al.*,1989). When these experiments were attempted with RRV using either BHK or Vero cell monolayers, the number of plaques formed following the temperature shift was low and unpredictable with respect to virus input. In addition it was difficult to maintain the monolayers intact so that plaques could be counted and thus repeatable results were not obtained (data not shown).

3.3.12 Binding assays using radiolabelled RRV T48 on BHK monolayers

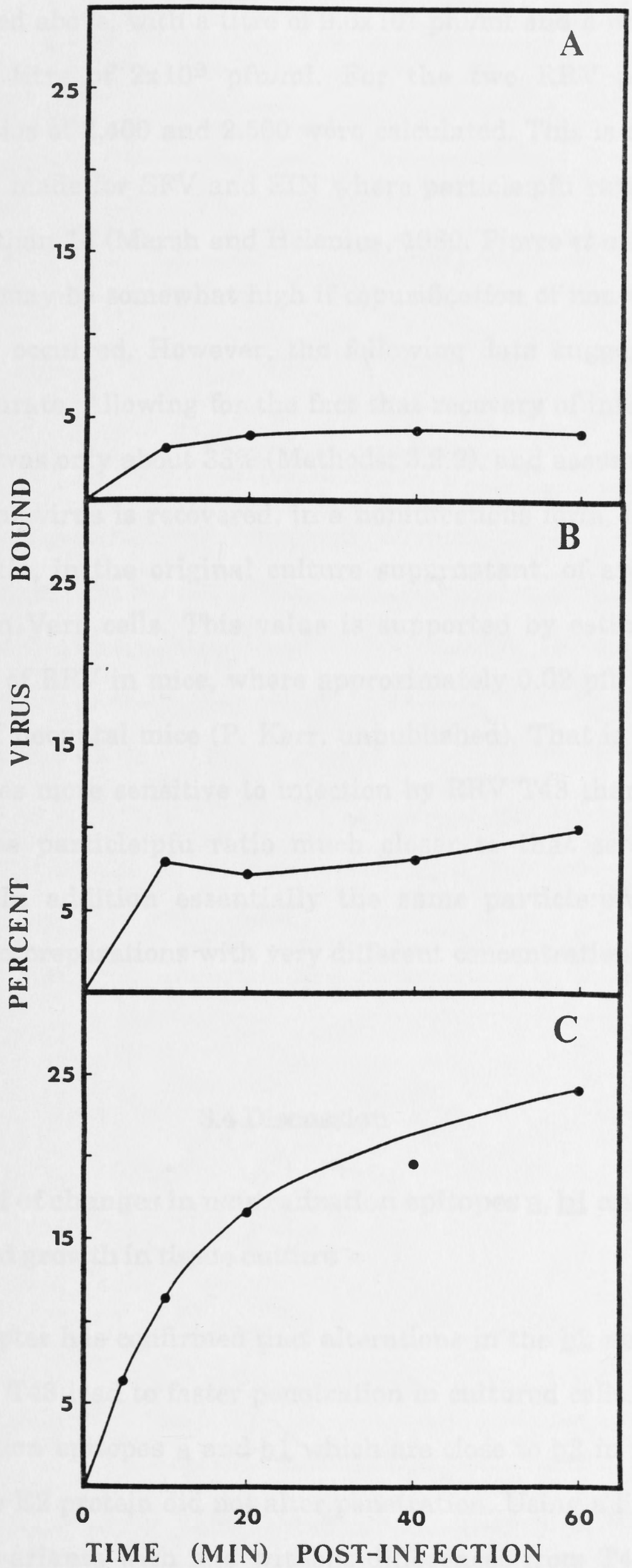
To overcome the difficulties involved in measuring the rate of RRV uptake by plaque formation, binding assays using [^{35}S]-methionine labelled RRV T48 were attempted (Methods; 3.2.10). At 4° and 20° the maximum amount of virus bound after washing was 4% and 10% respectively. There was little increase in binding between 10 and 60 min (Fig 3.13). Increasing the pfu:cell ratio did not increase the proportion of virus bound (data not shown). In contrast to the results at 4° and 20°, at 37° there was a progressive uptake of virus over a 60 minute period with about 25% of virus cell associated by 60 min. Unfortunately at this temperature the assay cannot distinguish between rate of virus binding and rate of virus entry into cells.

To examine the possibility that there was a very large amount of noninfectious virus competing for receptors in the binding assays described above, an estimate of particle:pfu ratio was made (Methods; 3.2.9). Two independently purified preparations of RRV were used: the T48

Figure 3.13

Kinetics of binding of [^{35}S]-methionine labelled RRV T48 to BHK cells at different temperatures

RRV T48 labelled with [^{35}S]-methionine was allowed to attach to BHK cell monolayers (pfu:cell~1) at either 4° (Fig a), room temperature (~20°, Fig b) or 37° (Fig c). At the indicated times monolayers were washed three times with HBSS (pH 7.2) at the same temperature as attachment. After draining, monolayers were dissociated in NP40 (1%) and the bound radioactivity determined by liquid scintillation counting. Virus bound is expressed as a percentage of the input number of virus cpm.


A
B
C
25
15
5
25
15
5
25
15
5
PERCENT
VIRUS BOUND
0
20
40
60
TIME (MIN) POST-INFECTION

preparation used above, with a titre of $9.6x10^7$ pfu/ml and a preparation of Tv161 with a titre of $2x10^9$ pfu/ml. For the two RRV preparations particle:pfu ratios of 2,400 and 2,500 were calculated. This is much higher than estimates made for SFV and SIN where particle:pfu ratios are often quoted as less than 10 (Marsh and Helenius, 1980, Pierce *et al.*,1974). This value for RRV may be somewhat high if copurification of non-viral protein with the virus occurred. However, the following data suggest that it is reasonably accurate. Allowing for the fact that recovery of infectious virus in purification was only about 33% (Methods; 3.2.9), and assuming that the remainder of the virus is recovered, in a noninfectious form, this leaves a particle:pfu ratio, in the original culture supernatant, of approximately 800 for RRV in Vero cells. This value is supported by estimates of the infectious dose of RRV in mice, where approximately 0.02 pfu of RRV T48 will kill 50% of neonatal mice (P. Kerr, unpublished). That is mice of this age are 50 times more sensitive to infection by RRV T48 than Vero cells. This brings the particle:pfu ratio much closer to that seen for other alphaviruses. In addition essentially the same particle:pfu ratio was obtained for two preparations with very different concentrations of virus.

3.4 Discussion

3.4.1 The effect of changes in neutralization epitopes a, b1 and b2 on RRV penetration and growth in tissue culture

This chapter has confirmed that alterations in the b2 neutralization epitope of RRV T48 lead to faster penetration in cultured cells. Changes in the neutralization epitopes a and b1 which are close to b2 in the primary structure of the E2 protein did not alter penetration. Using a limited range of geographic variants with and without differences from T48 in the b2 epitope it was demonstrated that all variants had the fast penetration type

therefore this could be regarded as normal and T48 as slowly penetrating. This reasoning fits well with the observation that any of the amino acid alterations in the b2 epitope gave fast penetration suggesting that b2 interacts with other areas on the E2 protein involved in cell attachment and entry and that changes in this region are altering determinants involved in penetration.

All of the mAb T1E7 selected variants used in this study had the fast penetration phenotype. Most of the assays described in this chapter were performed with Tv1 which has an Asp→Asn alteration at amino acid 246, this introduces a potential glycosylation site into the protein which, if utilized, may explain the mAb T1E7 resistance of this variant and of Tv161 which has the same change in epitope b2 (Vrati *et al.*,1988). The other b2 variants have quite different amino acid alterations; Tv2: Arg251→Ser and Tv5: Asp246→Val; Tv3 was not used here but has also been shown to have fast penetration in BHK cells (Vrati, 1986), it has a Thr248→Pro change in E2 (Vrati *et al.*,1988). Thus a positively charged amino acid changing to a hydrophilic or a negatively charged amino acid changing to a hydrophobic both produced a similar alteration in penetration rates to the Asp246→Asn change.

There does not appear to be any structural pattern in these amino acid alterations, it seems that an alteration in epitope b2 away from the T48 sequence will increase penetration rate. Epitope b2 occurs in an extremely hydrophilic region of E2 and thus is likely to be on the surface of the protein (Vrati *et al.*,1988). The introduction of bulky carbohydrate molecules, hydrophobic side chains, prolines or charge changes could influence local protein structure and may affect neighboring functional sites.

When further mAb T1E7 resistant variants of RRV T48 were selected similar changes to those reported by Vrati *et al.* (1988) were found.

This may imply some limitation to the changes acceptable at epitope b2 or may relate to the stringency of the screening assay for T1E7 resistance. Single nucleotide changes in the amino acid 246 codon could produce seven different amino acids, only one of which is conservative (Asp→Glu). A variant with this change would probably not be detected as resistant to T1E7 in the high stringency screening assay used. At the 251 codon six amino acid changes are possible from single base changes, two of these (Arg→Ser and Arg→Thr) were found in the present study. The only conservative change possible is Arg→Lys and this is seen in a number of natural isolates of RRV (Faragher *et al.*,1988; Burness *et al.*,1988; Vrati, 1986; C. Fernon, R. C. Weir and L. Dalgarno, unpublished).

Other fast penetrating variants of alphaviruses have been selected. Baric *et al.* (1981) selected a variant of SIN for rapid release from BHK cells and showed that this was associated with rapid penetration and replication in the line of BHK cells used for selection but not other cell lines, including other BHK lines. Russell *et al.* (1989) selected variants from a separate strain of SIN for rapid penetration of BHK cells. Selection of VEE for accelerated penetration of BHK cells (Johnston and Smith, 1988) produced variants with a range of increased penetration rates. In all cases selection of rapidly penetrating variants coselected for virulence attenuation in mice. The selection protocols used in these experiments do not appear to be selecting for an increased rate of virus binding to cells. Both Baric *et al.* (1981) and Pence *et al.* (1990) reported that the changes in penetration rate were not associated with alterations in the rate of virus adsorption. While in the selection protocol of Olmsted *et al.* (1984), Johnston and Smith (1988) and Russell *et al.* (1989) virus was allowed to adsorb to cells at 4° prior to shifting temperature to 37° for one minute and then treating cells with trypsin to remove virions that had not been internalized.

Using a full length cDNA infectious clone of SIN, Polo *et al.* (1988) demonstrated that the coselected fast penetration and low virulence of SIN mutants were caused by a Ser→Arg change at E2 114, demonstrated by Davis *et al.*,(1986). Ser(114) occurs in a hydrophobic stretch of 14 amino acids which is postulated to be buried inside the E2 protein. The need to accomodate a large, charged side chain from Arg could cause substantial rearrangements potentially modulating external domains involved in tissue tropism, receptor recognition and antibody binding (Davis *et al.*,1986). Thus it is predicted that amino acid 114 may not actually be directly involved in penetration. Some fast penetrating SIN variants selected by Russell *et al.* (1989) differed from the E2 114 variants by having an extra glycosylation site at amino acid one of E2. This prevented the cleavage of PE2; mutant virions contained PE2 instead of E2, had the fast penetration phenotype and were no longer neurovirulent for mice. The presence of PE2 could substantially alter the conformation of the virus spike. No sequence data are available for the VEE fast penetrating mutants.

The increased penetration of RRV variants in tissue culture differs in several points from that seen with SIN. Increased penetration in SIN is specific for the selecting line of BHK cells (Baric *et al.*,1981). For RRV the fast penetration phenotype is also seen with Vero cells. Increased penetration rates in both SIN and VEE coselected for substantially reduced virulence in mice (Olmsted *et al.*,1984; Russell *et al.*,1989; Johnston and Smith, 1988). In the case of RRV, Vrati (1986) showed very small differences in virulence for only two epitope b2 variants (Tv2 and Tv5) in terms of their LD_{50} values for seven-day old mice. None of the RRV b2 variants had faster replication in tissue culture which was a feature of the SIN variants (Baric *et al.*,1981), although this property of the SIN variants may have been due to a separate mutation. Finally there is some evidence

that the difference in penetration of Tv1 compared to T48 is partly due to faster tight attachment to cells.

The slower replication of RRV b2 variants during the early stages of infection reported by Vrati (1986) was not demonstrated in either BHK or Vero cells in the current work. (It is possible that under certain conditions, for example cells of a particular passage level or particular batches of bovine serum such an effect might be apparent). Tv161 grew less efficiently than the other virus types in Vero cells but this virus has three amino acid changes in the E2 glycoprotein and may not be well adapted to Vero cells. There was no evidence that changes in the b2 epitope had an effect at stages of cell entry other than attachment and binding to the cell surface, translocation and receptor mediated endocytosis.

3.4.2 The penetration of natural and laboratory derived strains of RRV in BHK cells

The NB5092 variant of RRV showed fast penetration kinetics in both BHK and Vero cells, as was predicted from its resistance to mAb T1E7. Similarly a mouse passage selected mutant of NB5092 (NB0/10/7; Meek *et al.*,1989) which is altered from NB5092 within the b2 epitope (Lys251→Asn) also had the fast penetration phenotype. This suggested that the substitution at this site is either not critical or that other amino acid changes in E2 and possibly E1 determine penetration rate for NB5092.

Four further natural isolates of RRV were examined in penetration assays, two of these, F9073 and BH38019, are altered at epitope b2 as demonstrated by mAb reactivity and sequence studies, the other two; PB629 and GG2227 had the same reactivity to mAb T1E7 as RRV T48 and were unchanged at epitope b2. All four of these isolates had fast penetration in BHK cells. Thus variations in the E1/E2 spike other than at epitope b2, must affect penetration rate.

The E2 sequences for F9073 (Burness *et al.*,1988) PB629, GG2227 and BH38019 (Vrati, 1986) have been determined. The region of E2 around the b2 epitope has been resequenced in the work reported here for PB629, GG2227 and BH38019 (Table 3.6). PB629 and GG2227 have an amino acid difference from T48 (Ile→Leu) at residue 67 (Vrati, 1986) which has not been resequenced in this study. This is a highly conservative change and appears unlikely to affect the biological properties of the virus, however, it cannot formally be excluded. F9073 has the same change at position 67 (Burness *et al.*,1988) and NB5092 is also altered from T48 (Ile→Met) at this residue (Faragher *et al.*,1988). A deletion of amino acids 55-61 of T48 E2 did not increase penetration rate in either BHK or Vero cells. NB5092 is also changed from T48 at 119 (Asp→Asn) which is very close to the 114 penetration change in SIN (Davis *et al.*, 1986), at residue 3 (Thr→Ile), at 251 (Arg→Lys) and at 302 (Glu→Val) (Faragher *et al.*,1988). In addition to the change at amino acid 67, F9073 differs from T48 at residues 132 (Asn→ Tyr), 203 (Cys→Trp) and 251 (Arg→Lys) (Burness *et al.*,1988). While the 251 residue remains the most likely to be involved in fast penetration it seems probable that the other changes are involved in stabilizing any protein structural alterations. It is also possible that a change exists in E1 in these variants which may influence protein structure and function. Although limited sequence analysis of the E1 gene for PB629, GG2227 and BH38019 did not demonstrate any coding changes.

3.4.3 The effect of pH on penetration of RRV T48, Tv1 and NB5092 in BHK cells

Experiments with penetration at a range of pH values indicated that Tv1 is very sensitive to alterations in pH when compared to T48 or the fast penetrating NB5092. These experiments did not differentiate between pH effect on the cell surface and on the viral proteins. However, it appears that

the penetration rate of NB5092 is much more stable at mildly acidic or basic pH than that of Tv1, suggesting that amino acids apart from those at b2 stabilize the interactions involved in penetration. One possibility that has not been examined here is that the more rapid entry and higher specific infectivity of Tv1 at pH 6.5 involves fusion of the virus at the cell plasma membrane as well as receptor mediated endocytosis especially since a large component of the extra penetration is within the first 10 min of infection. SFV fuses directly with the plasma membrane at pH 6.2, this implies that at this pH a structural alteration occurs in the viral spike glycoproteins which exposes the fusion domain (Kielian and Helenius, 1985). No data is available for RRV on the pH at which this occurs, however, the penetration rate of T48 is unchanged at 6.5, while NB5092 may be a little faster. Direct membrane fusion could also explain the higher specific infectivity of Tv1 at acid pH but does not alter the conclusion that the faster penetration at this pH implies a less stable E2 structure.

3.4.4 The time course of binding of radiolabelled RRV T48 to BHK cells

Only 4% of RRV bound to BHK cells at 4° and most binding occurred in the first 10 min pi. These results contrast with those reported for SIN and SFV on a variety of cell types where 15-90% of added virus was bound at this temperature (Pierce *et al.*,1974; Smith and Tignor, 1980; Fries and Helenius, 1979) and for SFV where the time for half maximal binding was approximately 30 min (Fries and Helenius, 1979).

Two points can be made about the behaviour of RRV in these assays: (1) at 4° it is possible that few virions are tightly bound, as receptor recruitment by translocation in the plasma membrane may be inhibited. However, Birdwell and Strauss (1974) have demonstrated that in chick cells such receptor mobility may occur at 4°. At room temperature about twice as much virus was bound but attachment was still essentially

complete within 10 min. (2) At 36° virus continued to attach to cells over the 60 minute period of the assays. This suggested that either receptor recruitment and tighter binding, together with internalization, were occurring thus making the virus difficult to remove by washing and that this was a prolonged process, or that greater receptor availability occurred at this temperature perhaps because of receptor recycling, implying that the number of receptors on the cell surface for RRV was very limited. Particle to pfu ratios indicated that a large amount of noninfectious RRV was competing for binding sites on cells. Numbers of binding sites for SIN and SFV have been estimated as between $5x10^4$ and $1.5x10^6$ (Birdwell and Strauss, 1974; Smith and Tignor, 1980; Fries and Helenius, 1979). If comparable numbers of binding sites were available for RRV, noninfectious virus is unlikely to have inhibited binding. These experiments were preliminary attempts to develop a cell binding assay for RRV, further work is needed to determine whether higher levels of RRV binding can be obtained with other strains of RRV. SIN or SFV should also be used as positive controls.

3.4.5 Conclusions and further work

This work has established that penetration rate of RRV is affected by epitope b2 but not epitopes a and b1. The mechanism of this is not understood. In a simple model, penetration of alphaviruses can be envisaged as a four step process. Initial, non-specific association of virions with the cell surface is followed by specific receptor binding and recruitment of additional receptors. Binding is virtually irreversible. Translocation of the virus-receptor complex to coated pits then occurs and finally endocytosis (Kielian and Helenius, 1986). An increase or decrease in penetration rate could result from changes at any of these steps. It is tempting to speculate that the changes at epitope b2 leading to fast penetration are increasing receptor affinity of the virus, especially as fast

penetration of Tv1 appears to involve an increased rate of attachment. Furthermore epitope b2 is a neutralization site and while little is known of the mechanism of RRV neutralization it is possible that mAb T1E7 acts by blocking virus binding to a receptor molecule on the cell.

Further work could be done to develop cell binding assays for RRV using fast entry types such as Tv1 and NB5092. This would allow testing of the hypothesis advanced in this discussion that changes in epitope b2 lead to faster penetration by increasing the rate of receptor binding. Use of an infectious centre assay for measuring penetration could also overcome the problems found with adsorbing virus at 4° prior to performing a penetration assay (Johnson and Smith, 1988). Additional sequencing studies of E1 for isolates such as PB629 and GG2227 could also define amino acids involved in penetration. Another set of experiments would be to select variants of RRV T48 and NB5092 for fast penetration using the protocol of Olmsted *et al.* (1984). An interesting experiment would be to passage T48 and Tv1 together in BHK cells and examine whether Tv1 became dominant, that is whether the difference in penetration rate would provide a competitive advantage in tissue culture. Such work should lead to better definition of the regions of RRV involved in cell entry and may enable strategies for isolating cell receptors to be developed.

Chapter Four

The selection of genetic variants of RRV by passaging in avian cells and the analysis of three selected variants with altered antigenic and biological properties

4.1 Introduction

Passaging in tissue culture has been used to select attenuated strains of VEE (Berge *et al.*,1961) and other viruses such as yellow fever (Theiler and Smith, 1937) for use as vaccines. In addition passaging RRV in tissue culture selected for reduced virulence in mice (Taylor and Marshall, 1975a). It appears that variants, either pre-existing or arising by mutation, that replicate more efficiently than the wild-type, in the particular tissue culture system, outgrow the wild-type to become the dominant population. In some cases this leads to reduced efficiency of replication or altered tissue tropism in the original host and thus attenuation.

Little is known of the molecular mechanism of selection in tissue culture. Changes could occur at the level of structural or nonstructural proteins or in untranslated regulatory regions of a virus. The results of several passaging studies with alphaviruses are consistent with the idea that adaptation to new cell types may involve selection for enhanced cell binding and entry, resulting from changes in the surface proteins of the virus (see Chapter 1). Thus mouse plasmacytoma cell adapted SIN had an increased negative charge on the envelope glycoproteins of the virus associated with enhanced binding to these cells (Symington and Schlesinger, 1978). VEE attenuated by cell passage to produce the vaccine strain, TC83, had only one nucleotide alteration outside the envelope protein genes (Kinney *et al.*,1989). Selection for rapid penetration of SIN in BHK cells involved a single amino acid change at position 114 of E2 which was also responsible for attenuation in mice (Davis *et al.*,1986; Polo *et al.*,1988).

The studies described in Chapter 3 showed that single amino acid alterations in the b2 epitope of RRV E2 can alter virus entry kinetics in

BHK and Vero cells. This suggested that a determinant on the E2 protein may be involved in cell attachment or receptor recognition. By passaging RRV in avian cells it was planned to select variants with adaptations to these cells and identify determinants on the viral glycoproteins involved in cell tropism. Using a cell type to which RRV is not biologically adapted (see below) provided the potential for a strong selection pressure while a low number of passages limited background changes.

Avian cells were selected because serological surveys and attempts to isolate RRV had indicated that birds were unlikely to be involved in the epizootology of this virus (Doherty *et al.*,1966; Gard *et al.*,1973; Marshall *et al.*,1982a). There is only one report of the isolation of RRV from birds (Whitehead *et al.*,1968) and RRV replicated poorly in birds following experimental infection (Whitehead, 1969; Gard, 1970). This is in contrast to the biology of some other alphaviruses, such as SIN, WEE and EEE, for which birds are important hosts (Chamberlain, 1980). Chick embryo fibroblasts (CEFs) have not been routinely used with RRV in laboratory studies so there was very limited data on the growth of RRV in these cells. RRV field isolates from Nelson Bay did not form plaques on primary chick embryo fibroblast (CEFs) monolayers when first isolated, however, some plaque formation was noted following two passages of the virus in suckling mouse brain (Gard, 1970).

In this chapter the development of conditions for passaging RRV in chick embryo fibroblasts is initially described. Following five passages in these cells, three independent clones of RRV were selected and characterized by antigenic analysis, growth in mice and nucleotide sequencing. Variants were altered at either amino acid 4 or 218 of E2. Those altered at 218 had attenuated growth in mice.

4.2 Materials and Methods

4.2.1 Virus Stocks

Plaque purified stocks of RRV NB5092 were produced from single plaques picked from Vero cell monolayers and amplified once in BHK cells to give three independent plaque purified stocks designated: NB1/0, NB2/0 and NB3/0. Stocks were titred by plaque assay on Vero monolayers. Production of plaque purified stocks of RRV T48 has been described in Chapter 3. Sindbis virus (HR, large plaque strain) was obtained from Dr. J.H. Strauss (California Institute of Technology, Pasadena, California).

4.2.2 Tissue culture

Primary chick embryo fibroblasts (CEFs) were prepared from 4-12 embryos between 9 and 10 days old. Embryos were removed from the eggs into cold PBS using sterile technique. The heads and feet were removed and the bodies transferred to fresh PBS prior to homogenization through a 10ml disposable syringe. After washing twice with ice-cold PBS the tissue fragments were digested with 0.25% trypsin in PBS, 7ml / embryo (5 min, 37°, with constant stirring). The supernatant was decanted, through four layers of gauze, into a 50ml centrifuge tube containing 1/10 volume of growth medium (M199 LAH+10% BS) on ice. Fresh trypsin was added to the remaining tissue fragments and the digestion repeated; this process was then performed a third time.

Cell suspensions were centrifuged (1600rpm, 4°, 15 min.) the supernatant discarded and the cells resuspended in growth medium; the cell suspension was made up to 50ml and the centrifugation repeated. Cells were resuspended in fresh growth medium and counted in a haemocytometer.

Primary cells (2-4x10^7) were seeded into 75cm^2 tissue culture flasks in M199 LAH+10% BS and grown to confluency. They were then harvested using trypsin/EDTA, seeded as secondary CEFs into 35mm dishes at 1-1.5x10^6 cells per dish in M199 LAH+10% BS and incubated at 36°/5% CO_2 until confluent. For passaging experiments secondary cells rather than primary cells were used so as to maintain the cells as constant as possible from batch to batch and to remove macrophages and other cells from the primary cells which could allow growth of RRV in non-fibroblast cells or could potentially modify fibroblasts by secreting cytokines.

Growth of Vero and BHK cells has been described in Chapter 2.

4.2.3 Passaging RRV in CEFs

Preliminary experiments in secondary CEFs were performed using the T48 and NB5092 strains of RRV at 36°. For passaging experiments confluent secondary CEF monolayers were infected with RRV (moi~0.5-1) and the culture medium harvested at 24 hours post-infection; 100µl of each supernatant was used to inoculate the next cell monolayer for up to five passages. Following experiments described in the results section the conditions were altered to growing virus at 30°/5% CO_2 for 40-48 hours and the NB5092 strain of RRV was used for passaging and the selection of passaged variants.

The fifth passage level stock of each passage series was plaque purified on Vero cell monolayers. Single plaques were amplified once in CEFs to produce seed stocks. Working stocks (ws) were prepared in CEFs from each seed stock and unless otherwise stated, all subsequent experiments were done with these working stocks. For antigenic analysis of virus from intermediate passage levels plaques were picked from Vero cell monolayers and amplified in BHK cells (see results).

4.2.4 Backpassaging CEF selected variants in BHK cells

Plaques from 1/5ws, 2/5ws and 3/5ws were picked from Vero cell monolayers and amplified in CEFs. Approximately 10^6 pfu of virus was used to infect BHK cell monolayers in 35mm dishes. Incubation was at 36°. The culture supernatant was harvested at the onset of cpe and 100µl used to infect the next monolayer in the series for a total of five passages.

4.2.5 Virus Nomenclature

RRV clones derived from NB5092 are identified by the number 1, 2 or 3 of the original NB5092 clone followed by the passage number ie NB1/5 refers to the fifth passage stock of NB5092 clone 1. Working stocks are identified by "ws" following the passage number. For backpassaging, plaques were picked from the working stocks and identified with a plaque number, thus NB1/5/1 refers to the first plaque picked from the NB1/5ws. These stocks are then further identified with the number of backpassages, thus 1/5/1BP5 refers to the first plaque from NB1/5ws after five backpassages in BHK cells.

4.2.6 Growth kinetics and RNA synthesis kinetics

These analyses were performed as described in Chapter 3.

4.2.7 Antigenic analysis

Antigenic analysis was performed using PRNA with mAbs and polyclonal antiserum; these were produced as described in Chapter 2. Two additional mAbs, T1D11 and T4D2 were used (Vrati *et al.*,1988).

4.2.8 Extraction of infected cell RNA

RNA was extracted from infected CEFs or BHK cells as described in Chapter 3.

4.2.9 Nucleotide sequencing

Nucleotide sequencing of CEF selected variants was as described in Chapter 3. RNA from NB5092 infected BHK cells was sequenced in parallel with the CEF variants. Most of the CEF variants nucleotide sequences were obtained using RNA extracted from RRV infected CEFs, however, in some regions it proved difficult to obtain clear sequence ladders using this RNA. These regions were sequenced using RNA extracted from infected BHK cells after it had been demonstrated that antigenic reversion did not occur on growth in these cells (see Results). The following oligonucleotide primers were used:

```
3'-UT/3853, TCTATTTGTCTACTT;    E1 3556, CGCCGTGGAAAAGTG;
E1 3178,    GTATGGCACATGCAC;     E1 2752, TCCACCCCACATGAA;
E1 3386,    ACCTGGCAGCTCAGG;     6K 2472, TGCGGAGGCTCCCAG;
6K 2347,    GGTTTTGTTCTCGTC;     E2 1987, GATGATGCGCTCAGA;
E2 1632,    TTGTACCTGATAGTC;     E2 1452, ACTTCTCTCTACCCACC;
E2 1225,    GGGCGTGGGTACCTG.
```

Primer DNA sequences are written in the 5'-3' direction. Priming sites are numbered from the 5' end of NB5092 26S RNA, (Faragher *et al.*,1988)

The primers at the 3' end of the E1 and E2 genes produced considerable cross-banding. Efforts were made to resolve these using 7-deazaGTP (Boehringer-Mannheim, West Germany) to replace dGTP in the incubation and chase mixtures (Barr *et al.*,1986), this was succesful in some instances but did not resolve the majority of cross-bands. Terminal deoxynucleotide transferase (Pharmacia LKB Biotechnology, Uppsala, Sweden) was also used (De Borde *et al.*,1986), together with a range of primer/template ratios and annealing temperatures. A major cross band at nucleotide 3437 caused problems finishing the sequence of E1. In

addition using the 2350 primer at the 3' end of E2 it was not possible to read the 3' terminal 120 nucleotides of the NB2/5ws sequence despite repeated attempts. These particular problems were largely overcome by using two new primers, E1/3386 and 6K/2472 but 55 nucleotides (3382-3437) of NB1/5ws E1 were not obtained. Where the cross-banding structure was the same for NB5092 as for the variants then the sequence was assumed to be unchanged. There were sometimes problems obtaining long (400-450 nucleotide) runs of sequence from CEF RNA, which may reflect high levels of ribonuclease in CEFs leading to fragmented viral RNA. For regions where this was a difficulty final sequence was obtained using IC/RNA extracted from BHK cells.

4.2.10 Temperature shift experiments

Duplicate monolayers of CEFs were infected with NB5092, (moi 0.51); adsorption was at either 36° or 30° for 60 min. At the end of this time the monolayers were washed twice (HBSS, pH7.2) and either shifted to the other temperature or allowed to continue at the original temperature; growth medium at the appropriate temperature was added. Growth samples were collected at 1, 24 and 50 hours pi and EV titres determined on Vero cell monolayers.

4.2.11 Growth and tissue tropism of CEF selected variants in mice

Litters of neonatal, Swiss White, outbred mice (<24 hours old) were randomized and fostered back onto mothers in litters of 10-12. For each of NB1/5ws, NB2/5ws, NB3/5ws and NB5092 three litters of mice were injected subcutaneously (sc) with 100 pfu of virus in HBSS. At 24 hour intervals three mice from each group were killed by exsanguination while anaethesized with ether. Blood was collected using heparinized capillary tubes after opening the thoracic cavity with scissors. As much blood as possible was removed from the mice to minimize the effects of viraemia on

virus levels in the tissues. Brains were removed intact and the hind leg and pelvic muscle tissue collected. For this, the skin over the pelvis and hind legs was removed and the leg below the stifle joint discarded, thus some of the femur was included. Tissues were collected into weighed tubes on ice and stored at -70° prior to processing.

Brains were homogenized in glass, hand-operated homogenizers followed by sonication (3 x 5 sec at an amplitude of 10 mA). For muscle tissue a motor-driven teflon-glass homogenizer was used followed by sonication, as above. All operations were performed on ice and dilution was in ice cold HBSS (pH 7.2) to produce 10% w/v suspensions. Blood was diluted to 10% v/v. Virus in each tissue was measured by duplicate plaque assay on Vero cell monolayers. Statistical analysis was by analysis of variance for blood and muscle virus titres. This was performed by Mr Ross Cunningham, Department of Statistics, The Faculties, Australian National University.

Brain titres were variable and to ensure that quantitative recovery of NB5092 from 10% brain homogenates was achieved, the following control experiment was performed. Duplicate samples of a mouse brain homogenate (10% w/v in HBSS, pH 7.2) with no detectable virus on plaque assay, were inoculated with approximately $8x10^6$ pfu of NB5092. One of the samples was clarified by centrifugation and the supernatant transferred to a fresh tube, the other was left unclarified. Both tubes were then allowed to stand on ice for 60 min at room temperature, frozen at -70° overnight and then thawed and plaque assayed. Full recovery of virus was made from both samples, indicating that there was no loss of virus infectivity during processing.

4.2.12 Virulence assay of NB5092 and NB1/5 ws in neonatal mice

To compare the virulence of NB5092 and the CEF selected variant NB1/5ws, randomized litters of neonatal mice (<24 hours old) were injected sc with 10^2, 10^3, 10^4 or 10^5 pfu of virus diluted in HBSS and observed daily for clinical symptoms and deaths. Clinical symptoms were graded as; (-): no symptoms; (+/-): mild hind leg weakness; (+): dragging one or both hind legs.

4.2.13 Hydropathy profiles and protein secondary structure predictions

The computer analyses of protein secondary structure predictions and hydropathy profiles were done using the PeptideStructure program and presented graphically using PlotStructure (Jameson and Wolf, 1988; Wolf *et al.*,1988).

4.3 Results

To select avian cell adapted variants of RRV attempts were made to grow and passage strains of RRV in secondary chick embryo fibroblasts (CEFs). Preliminary growth experiments using RRV NB5092 and T48 in CEFs at 36° indicated that neither virus grew to high titres. Although in some experiments NB5092 appeared to replicate more efficiently than T48, on passaging both viruses were generally lost by the second or third passage. No evidence of cytopathic effect (cpe) was observed in any experiment with either strain of RRV in CEFs. In a series of experiments with T48, the use of high moi (50-100) to initiate passage series, the presence of AMD or the use of virus grown in mosquito cells did not increase the efficiency of replication of RRV in CEFs (data not shown).

4.3.1 Growth experiments and plaque assays on CEF monolayers

To examine replication of RRV in CEFs more rigorously, growth and viral RNA synthesis, at 36°, for RRV T48 and NB5092 were examined. SIN was used as a positive control (Fig 4.1). T48 maintained a constant EV titre from 3-23 hours pi. The NB5092 titre was constant for the first 11 hours pi and then showed a slight increase. There was no detectable increase in AMD-resistant RNA synthesis for either NB5092 or T48. SIN gave a strong peak of RNA synthesis between 5-7 hours post-infection (pi). It appeared from this experiment that RRV replication in CEFs was blocked at the stage of cell entry or RNA synthesis.

As there was no evidence of cpe in RRV infected CEFs, plaque formation by RRV was examined on CEF cell monolayers. Adsorption was at 30° or 36° for 60 min. Monolayers were incubated at 30° or 36° for 48 hours prior to staining. No plaques were observed with either RRV T48 or NB5092. SIN was used as a positive control and formed large plaques at both temperatures with twice as many at 30° as at 36°.

4.3.2 The effect of temperature on the growth of RRV T48 and NB5092 in CEFs

In an attempt to define conditions under which RRV could be succesfully passaged in CEFs the effect of temperature on RRV replication in CEFs was examined. In a preliminary experiment CEF monolayers were infected (moi~5) with either T48 or NB5092. Adsorption and incubation were at either 30° or 36°. NB5092 grew to a titre of 8.3×10^7 pfu/ml at 30° but the titre was reduced 20 fold at 36°. T48 grew poorly at 36° and at 30° gave similar EV titres to NB5092 at 36° (Fig 4.2). This experiment indicated that the replication of RRV in CEFs showed strain specific

Figure 4.1

Virus growth and AMD resistant RNA synthesis at 36° in CEFs infected with RRV T48, NB5092 or Sindbis virus

CEF monolayers in 35mm dishes were infected (moi~1) with RRV T48, NB5092 or Sindbis virus. Control monolayers were mock-infected with HBSS. Incubation was at 36°; growth samples were taken from the supernatants at the indicated times and assayed for EV titres by plaque formation on Vero cell monolayers (Fig 4.1a).

To measure the kinetics of viral RNA synthesis cells were pulsed at appropriate times pi for 2 hours with 0.5ml of EMEM containing [5-^{3}H]-uridine (10μCi/ml), AMD (5μg/ml). After incubation (2 hours, 36°) the medium was aspirated, monolayers washed twice with PBS (pH 7.2) and dissociated with 200μl of 1% SDS. Acid precipitable radioactivity of duplicate 50μl aliquots was determined by liquid scintillation counting. Mean values of the duplicate samples are plotted (Fig 4.3b), time points represent the midpoint of each labelling period.

SIN

NB5092

T48

mock-infected

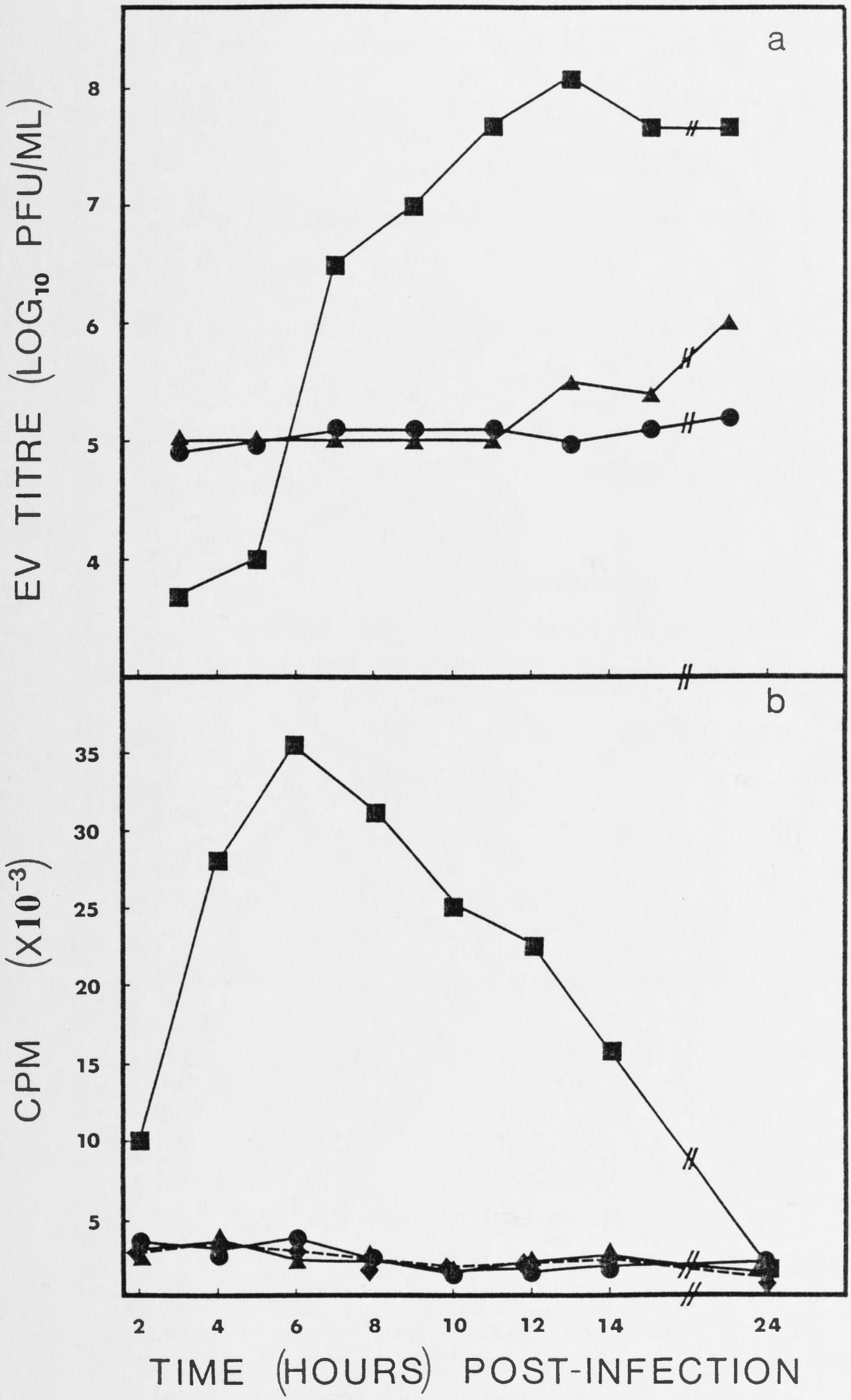
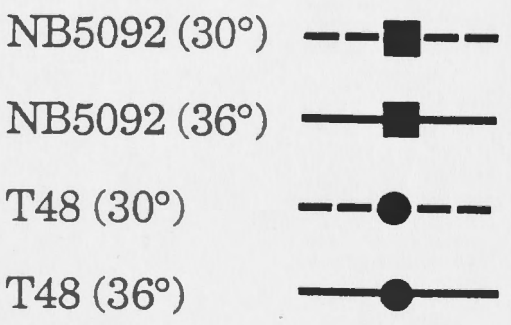
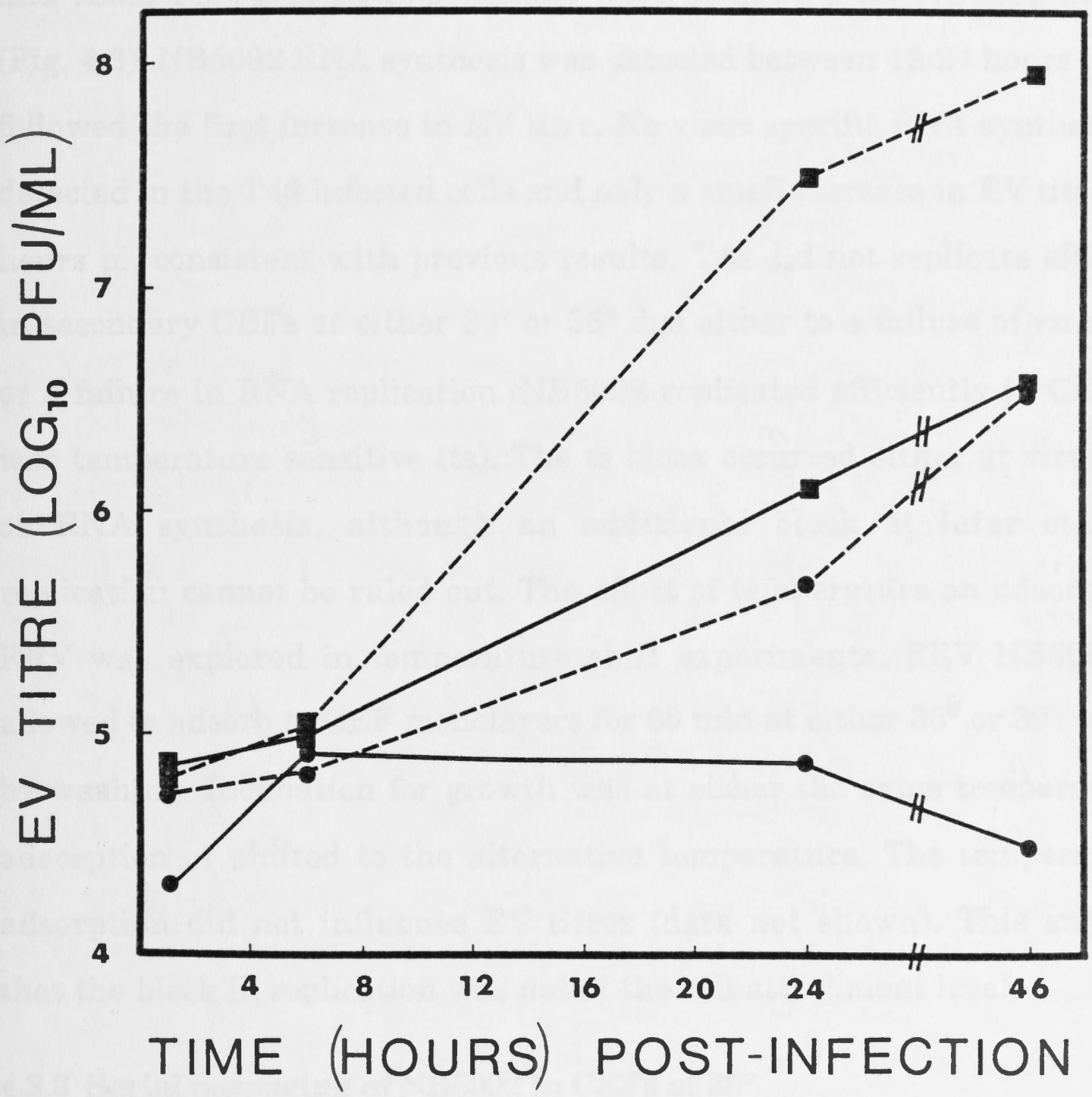

a
b
EV TITRE (LOG10 PFU/ML)
8
7
6
5
4
CPM (X10-3)
35
30
25
20
15
10
5
2
4
6
8
10
12
14
24
TIME (HOURS) POST-INFECTION

Figure 4.2

Growth of RRV T48 and NB5092 in CEFs at 30° or 36°

CEF monolayers in 35mm dishes were infected with either RRV T48 or NB5092 and duplicate monolayers incubated at 36° or 30°. Growth samples were collected at the indicated times and EV titres were determined by plaque formation on Vero cell monolayers at 36°.




EV TITRE (LOG10 PFU/ML)
8
7
6
5
4
4
8
12
16
20
24
46
TIME (HOURS) POST-INFECTION

differences and was temperature sensitive. There was no evidence of cpe in CEF monolayers infected with NB5092 at 30°.

To further characterize the growth of RRV in CEFs at 30°, EV titres and AMD resistant RNA synthesis were examined for NB5092 and T48 (Fig. 4.3). NB5092 RNA synthesis was detected between 12-24 hours pi. This followed the first increase in EV titre. No virus specific RNA synthesis was detected in the T48 infected cells and only a small increase in EV titre by 25 hours pi, consistent with previous results. T48 did not replicate efficiently in secondary CEFs at either 30° or 36° due either to a failure of viral entry or a failure in RNA replication. NB5092 replicated efficiently in CEFs but was temperature sensitive (ts). The ts block occurred either at virus entry or RNA synthesis, although an additional block at later stages in replication cannot be ruled out. The effect of temperature on adsorption of RRV was explored in temperature shift experiments, RRV NB5092 was allowed to adsorb to CEF monolayers for 60 min at either 36° or 30° followed by washing. Incubation for growth was at either the same temperature as adsorption or shifted to the alternative temperature. The temperature of adsorption did not influence EV titres (data not shown). This suggested that the block in replication was not at the cell attachment level.

4.3.3 Serial passaging of NB5092 in CEFs at 30°

To obtain variants of RRV that were selected by growth in CEFs, three independent clones of NB5092 were serially passaged in CEFs at 30° to produce three independent passage series. For each passage series initial moi was approximately one; culture medium was harvested at 48 hours pi for each passage and 100µl used to infect the next monolayer in the series. Five serial passages were performed in this manner.

The clones derived from NB5092 grew to high titres (~10^8 pfu/ml) in the first passage, consistent with previous results. However, in succeeding

Figure 4.3

Virus growth and AMD resistant RNA synthesis at 30° in CEF monolayers infected with RRV T48 or NB5092

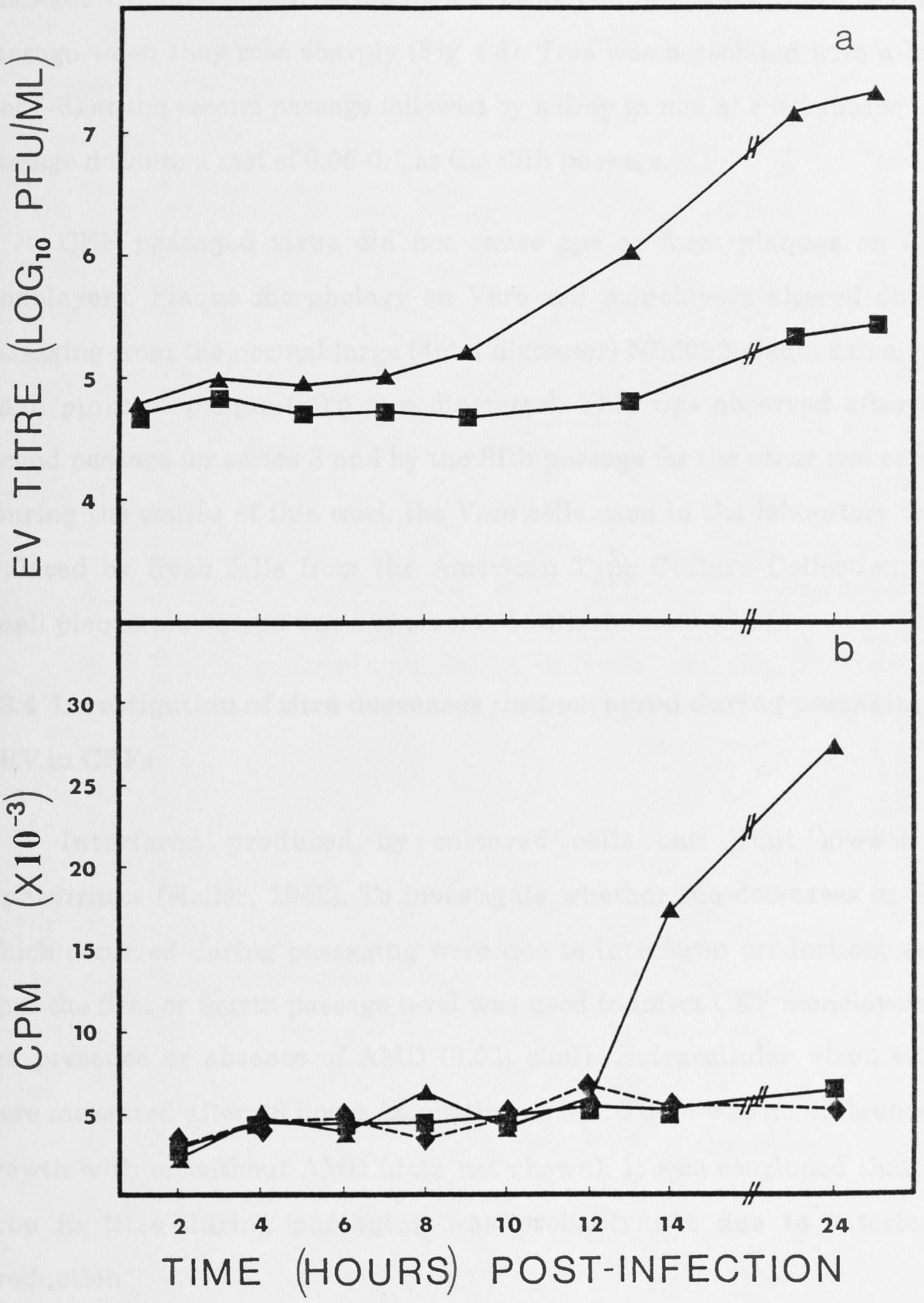
CEF monolayers in 35mm dishes were infected (moi~1) with RRV T48 or NB5092. Control monolayers were mock-infected with HBSS. Incubation was at 30°; growth samples were taken from the supernatants at the indicated times and assayed for EV titres by plaque formation on Vero cell monolayers at 36° (Fig 4.3a).

To measure the kinetics of viral RNA synthesis, cells were pulsed at appropriate times pi for 2 hours with 0.5ml of EMEM containing [5-^{3}H]-uridine (10μCi/ml), AMD (5μg/ml). After incubation (2 hours, 30°) the medium was aspirated, monolayers washed twice with PBS (pH 7.2) and dissociated with 200μl of 1% SDS. Acid precipitable radioactivity of duplicate 50μl aliquots was determined by liquid scintillation counting. Mean values of the duplicate samples are plotted (Fig 4.3b), time points represent the midpoint of each labelling period.

NB5092 

T48 

mock-infected 


a
b
EV TITRE (LOG10 PFU/ML)
7
6
5
4
CPM (X10-3)
30
25
20
15
10
5
2
4
6
8
10
12
14
24
TIME (HOURS) POST-INFECTION

passages virus titres fell, becoming as low as 10^5-10^6 pfu/ml, until the fifth passage when they rose sharply (Fig 4.4). This was associated with a high moi (~5) at the second passage followed by a drop in moi at each subsequent passage down to a moi of 0.05-0.1 at the fifth passage.

CEF passaged virus did not cause cpe or form plaques on CEF monolayers. Plaque morphology on Vero cell monolayers altered during passaging from the normal large (4mm diameter) NB5092 plaques to a very small pinpoint plaque (<0.5 mm diameter). This was observed after the second passage for series 3 and by the fifth passage for the other two series. [During the course of this work the Vero cells used in the laboratory were replaced by fresh cells from the American Type Culture Collection; the small plaque phenotype was not observed with the new cells].

4.3.4 Investigation of titre decreases that occurred during passaging of RRV in CEFs

Interferon produced by cultured cells can limit growth of alphaviruses (Heller, 1963). To investigate whether the decreases in titre which occurred during passaging were due to interferon production, virus from the first or fourth passage level was used to infect CEF monolayers in the presence or absence of AMD (0.01μg/ml). Extracellular virus titres were measured after 48 hours incubation at 30°. There was no difference in growth with or without AMD (data not shown). It was concluded that the drop in titre during passaging was probably not due to interferon production.

RNA synthesis and virus growth were examined in CEFs for NB5092, NB1/1 (NB clone 1 after the first passage) and NB1/5 (NB clone 1 after the fifth passage). AMD resistant RNA synthesis was first detected between 6-8 hours for NB1/5 and between 8-10 hours for NB5092. There was a consistently greater rate of incorporation of ^{3}H-uridine for cells infected

Figure 4.4

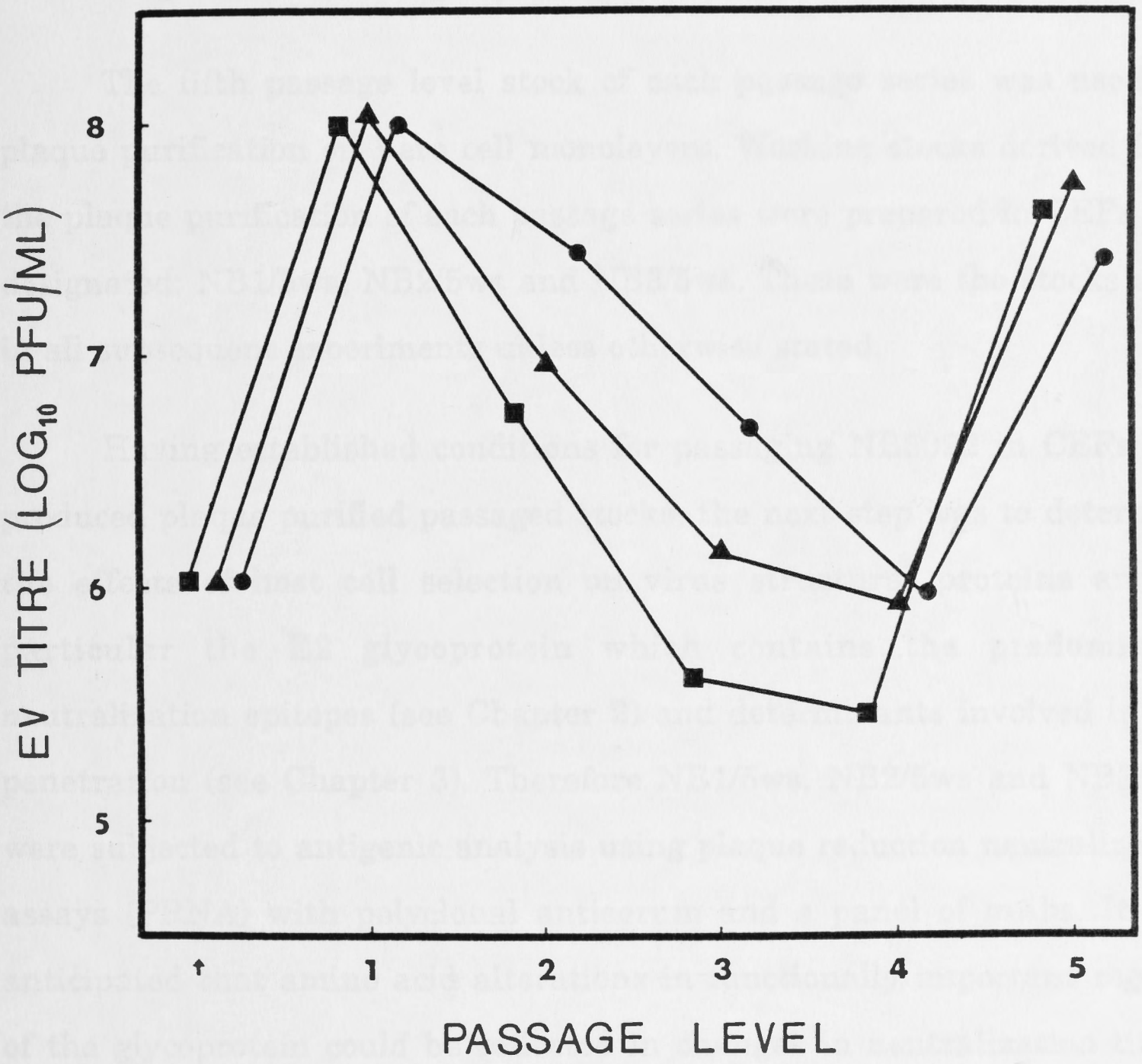
Extracellular virus titres of NB5092 clones during passaging in CEFs at 30°

Three independent clones of NB5092 were blind passaged five times in CEFs at 30°. Monolayers were in 35mm dishes; initial moi was approximately one. Supernatants were harvested at approximately 48 hours pi and 100µl of each supernatant was used to inoculate the next monolayer. Titres for each passage level were measured by plaque assay on Vero cell monolayers at 36°. The arrow represents the total initial inoculum of 10^6 pfu per monolayer.

Passage series 1 ■

Passage series 2 ▲

Passage series 3 ●


EV TITRE (LOG10 PFU/ML)
8
7
6
5
0
1
2
3
4
5
PASSAGE LEVEL

with NB1/5 compared to NB5092. RNA synthesis for NB1/1 was only marginally above that of the control cells. The lag period for EV NB1/5 was shorter than for NB5092 but EV titres were then similar up to 24 hours pi with NB5092 subsequently reaching a higher final titre. NB1/1 increased in EV titre at the same time as NB5092 but then grew considerably slower before reaching the same final titre as NB1/5 (Fig 4.5).

4.3.5 Antigenic analysis of plaque purified virus from CEF passage level 5.

The fifth passage level stock of each passage series was used for plaque purification on Vero cell monolayers. Working stocks derived from the plaque purification of each passage series were prepared in CEFs and designated: NB1/5ws, NB2/5ws and NB3/5ws. These were the stocks used in all subsequent experiments unless otherwise stated.

Having established conditions for passaging NB5092 in CEFs and produced plaque purified passaged stocks, the next step was to determine the effects of host cell selection on virus structural proteins and in particular the E2 glycoprotein which contains the predominant neutralization epitopes (see Chapter 2) and determinants involved in cell penetration (see Chapter 3). Therefore NB1/5ws, NB2/5ws and NB3/5ws were subjected to antigenic analysis using plaque reduction neutralization assays (PRNA) with polyclonal antiserum and a panel of mAbs. It was anticipated that amino acid alterations in functionally important regions of the glycoprotein could be reflected in changes in neutralization titres. The NB5092 stock from which clones 1, 2 and 3 were derived was used as the control virus after it was demonstrated that the unpassaged clones did not differ antigenically from each other, or from the parental NB5092 in PRNA with the panel of mAbs used (data not shown).

RRV NB5092, NB1/5ws, NB2/5ws and NB3/5ws were compared in PRNA using serial two-fold dilutions of polyclonal anti-RRV ascitic fluid.

Figure 4.5

Virus growth and AMD resistant RNA synthesis at 30° in CEF monolayers infected with RRV NB5092, NB1/5 or NB1/1

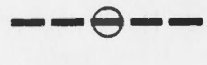
CEF monolayers in 35mm dishes were infected (moi~0.5) with RRV NB5092 or the CEF passaged populations NB1/1 or NB1/5. Control monolayers were mock-infected with HBSS. Incubation was at 30°; growth samples were taken from the supernatants at the indicated times and assayed for EV titres by plaque formation on Vero cell monolayers at 36° (Fig. 4.5a).

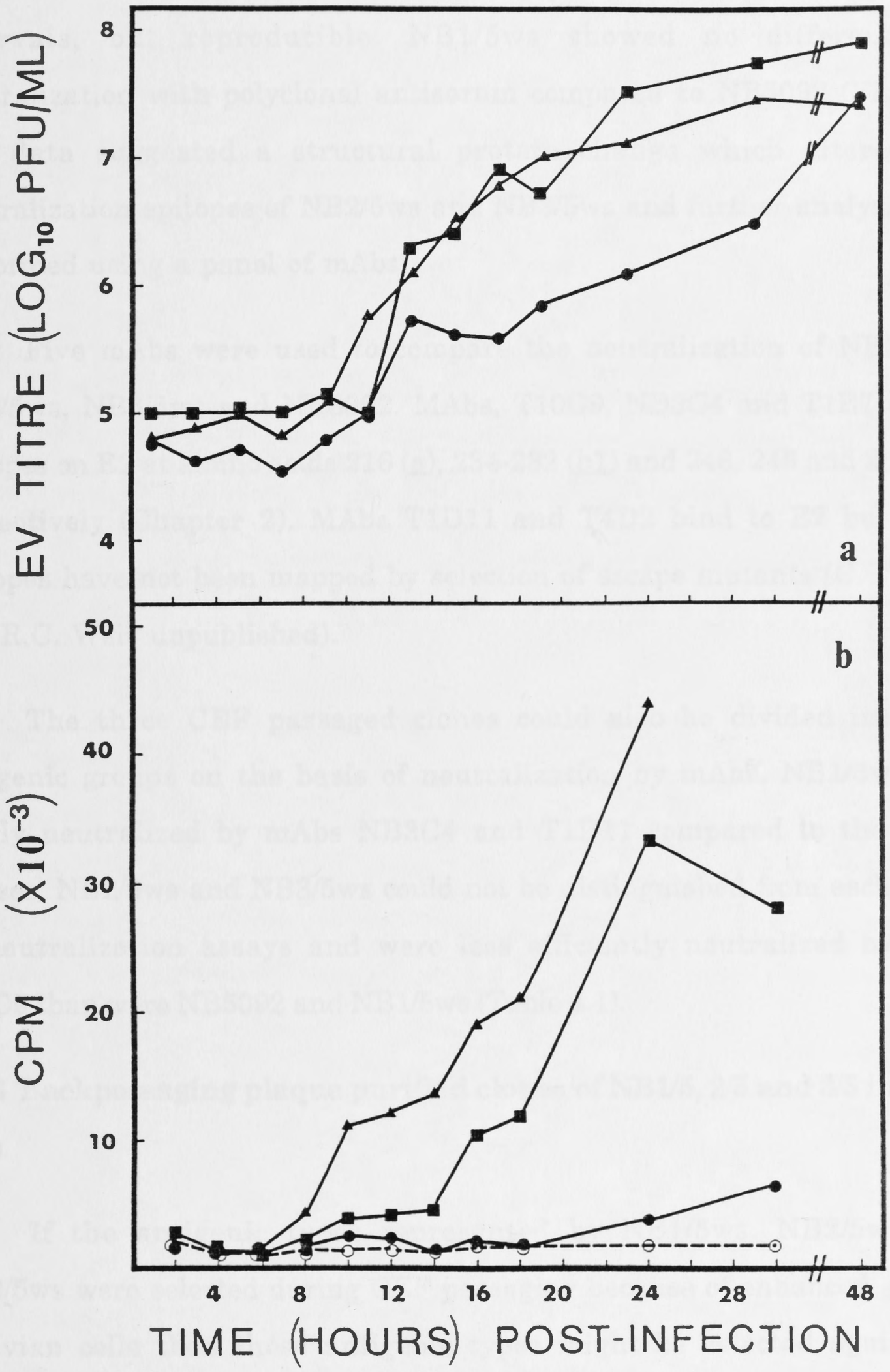
To measure the kinetics of viral RNA synthesis, cells were pulsed at appropriate times pi for 2 hours with 0.5ml of EMEM containing [5-^{3}H]-uridine (10μCi/ml), AMD (5μg/ml). After incubation (2 hours, 30°) the medium was aspirated, monolayers washed twice with PBS (pH 7.2) and dissociated with 200μl of 1% SDS. Acid precipitable radioactivity of duplicate 50μl aliquots was determined by liquid scintillation counting. Mean values of the duplicate samples are plotted (Fig. 4.5b), time points represent the midpoint of each labelling period.

NB5092 

NB1/5 

NB1/1 

mock-infected 


EV TITRE (LOG10 PFU/ML)
8
7
6
5
4
a
50
40
30
20
10
b
CPM (X10-3)
4
8
12
16
20
24
28
48
TIME (HOURS) POST-INFECTION

In two separate assays NB2/5ws and NB3/5ws were neutralized more efficiently than NB5092. The differences were small, less than two dilution intervals, but reproducible. NB1/5ws showed no difference in neutralization with polyclonal antiserum compared to NB5092 (Fig 4.6). The data suggested a structural protein change which altered the neutralization epitopes of NB2/5ws and NB3/5ws and further analysis was performed using a panel of mAbs.

Five mAbs were used to compare the neutralization of NB1/5ws, NB2/5ws, NB3/5ws and NB5092. MAbs, T10C9, NB3C4 and T1E7 define epitopes on E2 at amino acids 216 (a), 234-232 (b1) and 246, 248 and 251 (b2) respectively (Chapter 2). MAbs T1D11 and T4D2 bind to E2 but their epitopes have not been mapped by selection of escape mutants (C. Fernon and R.C. Weir unpublished).

The three CEF passaged clones could also be divided into two antigenic groups on the basis of neutralization by mAbs. NB1/5ws was poorly neutralized by mAbs NB3C4 and T1D11 compared to the other viruses. NB2/5ws and NB3/5ws could not be distinguished from each other in neutralization assays and were less efficiently neutralized by mAb T10C9 than were NB5092 and NB1/5ws (Table 4.1).

4.3.6 Backpassaging plaque purified clones of NB1/5, 2/5 and 3/5 in BHK cells

If the antigenic types represented by NB1/5ws, NB2/5ws and NB3/5ws were selected during CEF passaging because of enhanced growth in avian cells then these antigenic types might be selected against in mammalian cells. To examine the stability of the new antigenic types during passage in mammalian cells, plaques from NB1/5ws, NB2/5ws and NB3/5ws were picked from Vero cell monolayers and amplified in CEFs. One stock from NB1/5ws and two independent stocks from each of NB2/5ws

Figure 4.6

Neutralization of RRV NB5092 and the CEF passaged variants NB1/5, NB2/5 and NB3/5 by polyclonal anti-RRV serum

Neutralization of NB5092, NB1/5, NB2/5 and NB3/5 by polyclonal anti-RRV serum was measured by PRNA. Approximately 220 pfu of each virus stock were incubated with each dilution of antiserum for 60 min at 36°. Control virus was incubated with 1:400 normal ascitic fluid. Duplicate 100µl aliquots were plaque assayed on Vero cell monolayers and the percentage neutralization at each dilution calculated as the reduction in mean plaque numbers compared to the controls. The four viruses were assayed in parallel. The results of two independent PRNAs are shown in each panel as solid lines: assay 1, or broken lines: assay 2.

panel a NB5092

panel b NB1/5ws

panel c NB2/5ws

panel d NB3/5ws

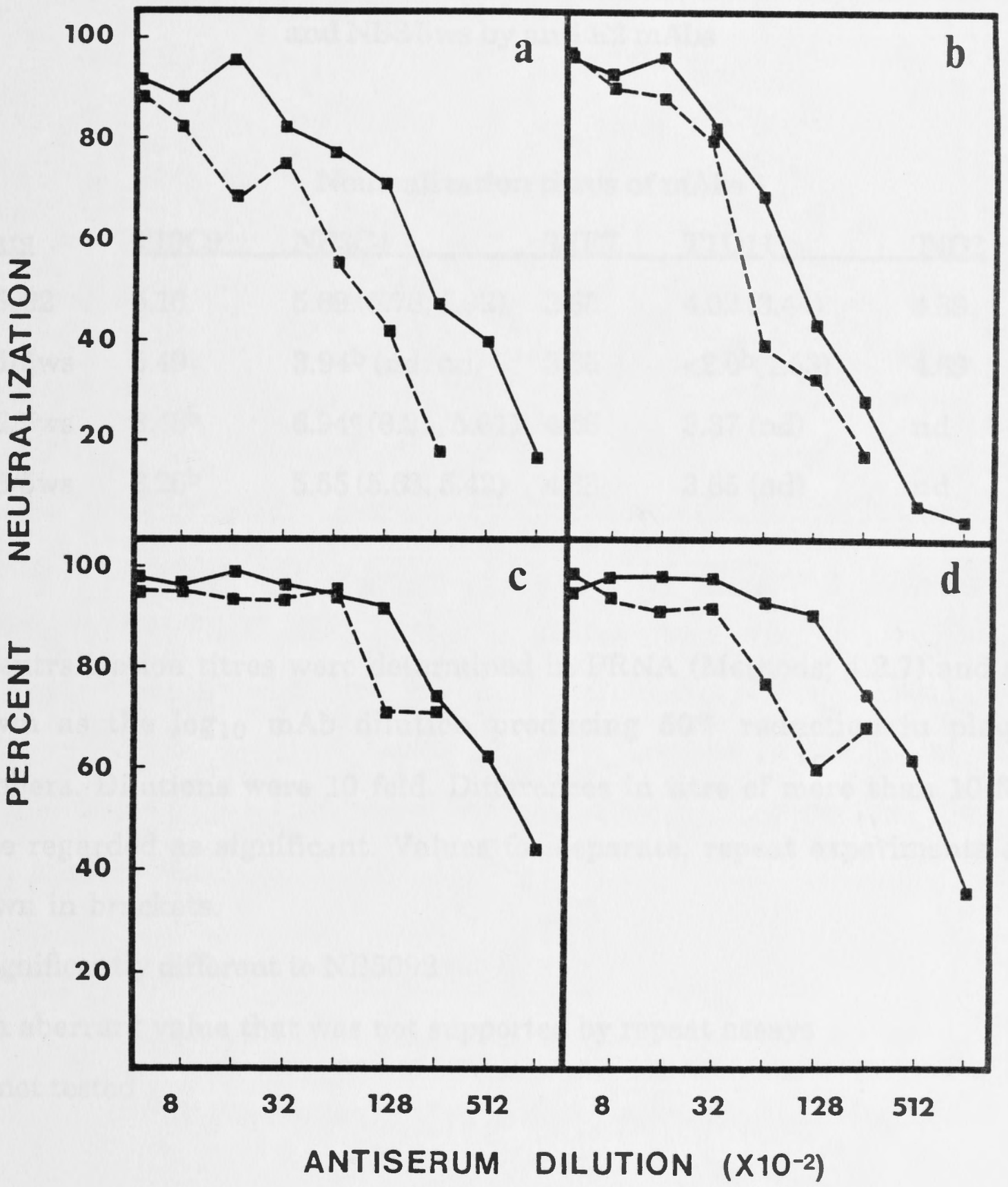

a
b
c
d
100
80
60
40
20
PERCENT NEUTRALIZATION
8
32
128
512
ANTISERUM DILUTION (X10⁻²)

Table 4.1

Neutralization of NB5092 and the CEF derived variants NB1/5ws, NB2/5ws and NB3/5ws by anti E2 mAbs

	Neutralization titres of mAbs [a]				
Virus	**T10C9**	**NB3C4**	**T1E7**	**T1D11**	**T4D2**
NB5092	5.10	5.69 (5.78, 5.92)	3.65	4.02 (3.44)	4.89
NB1/5ws	5.49	3.94[b] (nd, nd)	3.35	<2.0[b](1.53)	4.69
NB2/5ws	3.48[b]	6.94[c] (6.31, 5.61)	4.56	3.37 (nd)	nd
NB3/5ws	3.26[b]	5.55 (5.68, 5.42)	4.38	3.65 (nd)	nd

[a] Neutralization titres were determined in PRNA (Methods; 4.2.7) and are shown as the $\log_{10}$ mAb dilution producing 50% reduction in plaque numbers. Dilutions were 10 fold. Differences in titre of more than 10 fold were regarded as significant. Values for separate, repeat experiments are shown in brackets.

[b] significantly different to NB5092

[c] an aberrant value that was not supported by repeat assays

nd not tested

and NB3/5ws were then blind passaged five times in BHK cells (initial moi 0.5-1). Incubation was at 36°, supernatants were harvested at the onset of cpe and 100µl of each supernatant was used to initiate the next passage.

During backpassaging all stocks grew to high titres (>10^8 pfu/ml as measured on Vero cell monolayers) in BHK cells, at all passage levels and caused cpe (data not shown). They retained the small plaque phenotype. After the fifth passage the five, non-plaque purified, passaged populations were compared with the starting stocks in a PRNA against NB3C4 or T10C9 (Table 4.2). There was no significant difference in neutralization of passaged and unpassaged virus by these mAbs.

It was concluded that the antigenic changes defined by mAbs T10C9 and NB3C4 selected on passaging in avian cells were stable on backpassage in BHK cells. Passaging CEF adapted virus in BHK cells, even at high multiplicities of infection, did not lead to the decreases in titre that occurred on initial passaging in CEFs. In addition the viruses were all able to grow to high titres at 36° indicating that replication was not temperature sensitive in BHK cells.

4.3.7 The proportion of antigenic variants in CEF passage levels 2 and 3

Following repeated passage of NB5092 in CEFs, at least two independent antigenic types had arisen, these were defined by decreased sensitivity to neutralization by mAbs NB3C4 or T10C9. One type, resistant to mAb T10C9 was selected twice independently. These variants were plaque purified from a limit dilution of the fifth passage stocks and may or may not have been representative of the population. To examine at what passage level these variants could be detected and what proportion of the population they represented, a series of plaques was picked from passage levels 2 and 3 of each passaging series. These plaques were amplified in BHK cells (BHK cells were used since no antigenic reversion had occurred

Table 4.2

Antigenic analysis of CEF passaged antigenic variants of NB5092 after backpassaging in BHK cells

	Neutralization titre of mAbs [a]	
Virus [b]	**NB3C4**	**T10C9**
NB1/5/1	3.59	nd
1/5/1/BP5	3.75	nd
NB2/5/2	nd	3.10
2/5/2/BP5	nd	2.58
NB2/5/4	nd	2.73
2/5/4/BP5	nd	2.58
NB3/5/2	nd	2.44
3/5/2/BP	nd	2.51
NB3/5/4	nd	2.37
3/5/4/BP5	nd	2.22

[a] Neutralization titres are shown as the $\log_{10}$ mAb dilution producing 50% neutralization. Dilutions were 10 fold. Differences in titre of more than 10 fold were regarded as significant.

[b] Plaques of NB1/5ws, NB2/5ws, NB3/5ws, were picked and amplified in CEF cells. One clone of NB1/5ws, denoted NB1/5/1, and two clones of NB2/5ws and NB3/5ws, designated NB2/5/2, NB2/5/4, NB3/5/2,and NB3/5/4, were passaged five times in BHK cells (indicated as BP after the virus code). The final passage stocks were assayed in parallel with starting stocks in a PRNA against the mAb defining the antigenic type.

nd not tested

on backpassaging the CEF selected variants and CEFs were not routinely available) and stocks were analysed using PRNA with mAbs T10C9 and NB3C4.

For NB1/3 two of three isolates were resistant to NB3C4 indicating that the antigenic change had become dominant by the third passage. For passage series 2, three of four isolates examined at passage level 2 were resistant to mAb T10C9. The one sensitive isolate was also sensitve to NB3C4 indicating that it had the NB5092 phenotype. A similar result was obtained for isolates examined from the third passage level. In passage series 3, one of four isolates was resistant to mAb T10C9 at the third passage level. One of the sensitive isolates was also assayed for resistance to mAb NB3C4 and proved sensitive (Table 4.3).

Thus during the three passage series, three independent clones of NB5092 have generated two antigenic types independently and at apparently different rates. The clones were antigenically equivalent prior to passaging. Mixed population of antigenic variant and wild-type occurred but a mixture of the two antigenic types was not detected.

4.3.8 Antigenic analysis of the non-plaque purified fifth passage stocks.

To examine the dominance of the antigenic variants in the fifth CEF passage populations, the non-plaque purified stocks NB1/5, NB2/5 and NB3/5 were examined using a PRNA with mAbs NB3C4 and T10C9 and compared with the plaque purified stocks and NB5092 in the same assay (Table 4.4). The plaque purified and non-plaque purified stocks of NB1/5 were both increased in resistance to NB3C4, however, the plaque purified stock was 10 fold more resistant suggesting that wild-type NB5092 was still present at this level. For passage series 2 there was no difference between the plaque purified and non plaque purified fifth passage stocks and both were increased in resistance to mAb T10C9. Passage series 3 showed no

Table 4.3

[a]Single plaques picked from second and third level CEF passages were amplified in BHK cells and assayed for neutralization in PRNA using mAbs T10C9 and NB3C4. Dilution intervals were 10 fold. Differences in titre of greater than 10 fold were regarded as significant. All experiments were performed using NB1/5ws, NB2/5ws, NB3/5ws and NB5092 as controls. Sensitivity or resistance were assigned accordingly. Intermediate sensitivities were not detected.

[b] indicates the NB5092 clone, followed by the passage number from which the plaque was selected.

S sensitive

R resistant

nd not tested

Table 4.3

Summary of antigenic analysis of plaques from intermediate CEF passages of NB5092 [a]

		Monoclonal antibody	
Virus [b]	**plaque no.**	**NB3C4**	**T10C9**
NB1/3	3	R	nd
NB1/3	4	S	nd
NB1/3	8	R	nd
NB2/2	1	S	S
NB2/2	2	nd	R
NB2/2	3	nd	R
NB2/2	4	nd	R
NB2/3	1	nd	R
NB2/3	2	nd	R
NB2/3	3	nd	R
NB2/3	4	nd	S
NB2/3	5	nd	R
NB3/3	1	S	S
NB3/3	2	nd	S
NB3/3	4	nd	S
NB3/3	6	nd	R

Table 4.4

Neutralization assays of the non-plaque purified, fifth CEF passage virus stocks [a]

Virus	mAb T10C9	mAb NB3C4
NB5092	6.0 (S)	5.82 (S)
NB1/5ws	6.0 (S)	3.57 (R)
NB1/5np	6.0 (S)	4.57 (R*)
NB2/5ws	2.33 (R)	5.74 (S)
NB2/5np	2.37 (R)	5.47 (S)
NB3/5ws	2.41 (R)	5.71 (S)
NB3/5np	5.86 (S)	5.54 (S)

[a]Neutralization of NB1/5ws, NB2/5ws, NB3/5ws and the non plaque purified, fifth CEF passage stocks from which these were derived, was compared to NB5092 in a PRNA. Neutralization titres are shown as the $\log_{10}$ mAb dilution producing 50% neutralization for mAbs T10C9 and NB3C4. Ten fold dilution intervals were used throughout. Differences in titre greater than 10 fold were regarded as significant. S, sensitive to neutralization by the mAb; R, resistant to neutralization compared to NB5092; R*, intermediate resistance; np, non-plaque purified.

difference in neutralization titre between the non-plaque purified stock and the NB5092 control. Since plaque purified NB3/5 was resistant to mAb T10C9 it appeared that this antigenic type was a minority of the population. It was also apparent that non-plaque purified NB3/5 was sensitive to mAb NB3C4 thus it appeared that the NB1/5 antigenic type was not significantly represented in this population and that the NB5092 antigenic type was predominant.

4.3.9 Growth of RRV NB5092 and of CEF selected variants in CEFs

One possible approach to confirming a selective advantage of the selected antigenic variants in CEFs is to examine the early growth kinetics of the viruses in CEFs. This was done by infecting CEF monolayers in triplicate with NB1/5ws, NB2/5ws, NB3/5ws or NB5092 (moi~0.5) and taking growth samples over the first 25 hours post-infection. The infected monolayers were incubated at 30°.

NB5092 had a latent period of 7-9 hours, followed by a steady rise to a mean titre of $5x10^7$ pfu/ml. In contrast all the antigenic variants had a latent period of 11-13 hours (Fig 4.7). This experiment does not support the idea that selection of the antigenic variants in CEFs has been due to an early growth rate advantage. Interestingly the reverse appears to be the case with the selected virus growing slower than NB5092.

4.3.10 Growth in mice of NB5092, NB1/5ws, NB2/5ws and NB3/5ws

The NB5092 strain of RRV has been characterized extensively in mice (Mims *et al.*,1973; Murphy *et al.*,1973; Taylor and Marshall, 1975a). Unadapted NB5092 has low virulence for neonatal mice but on passaging in mice rapidly becomes virulent (Taylor and Marshall, 1975a; Meek *et al.*,1989). The increased virulence was accompanied by changes in the E2 glycoprotein (Meek *et al.*,1989). These changes were not essential for

Figure 4.7

Growth of RRV NB5092, NB1/5ws, NB2/5ws and NB3/5ws in CEFs at 30°

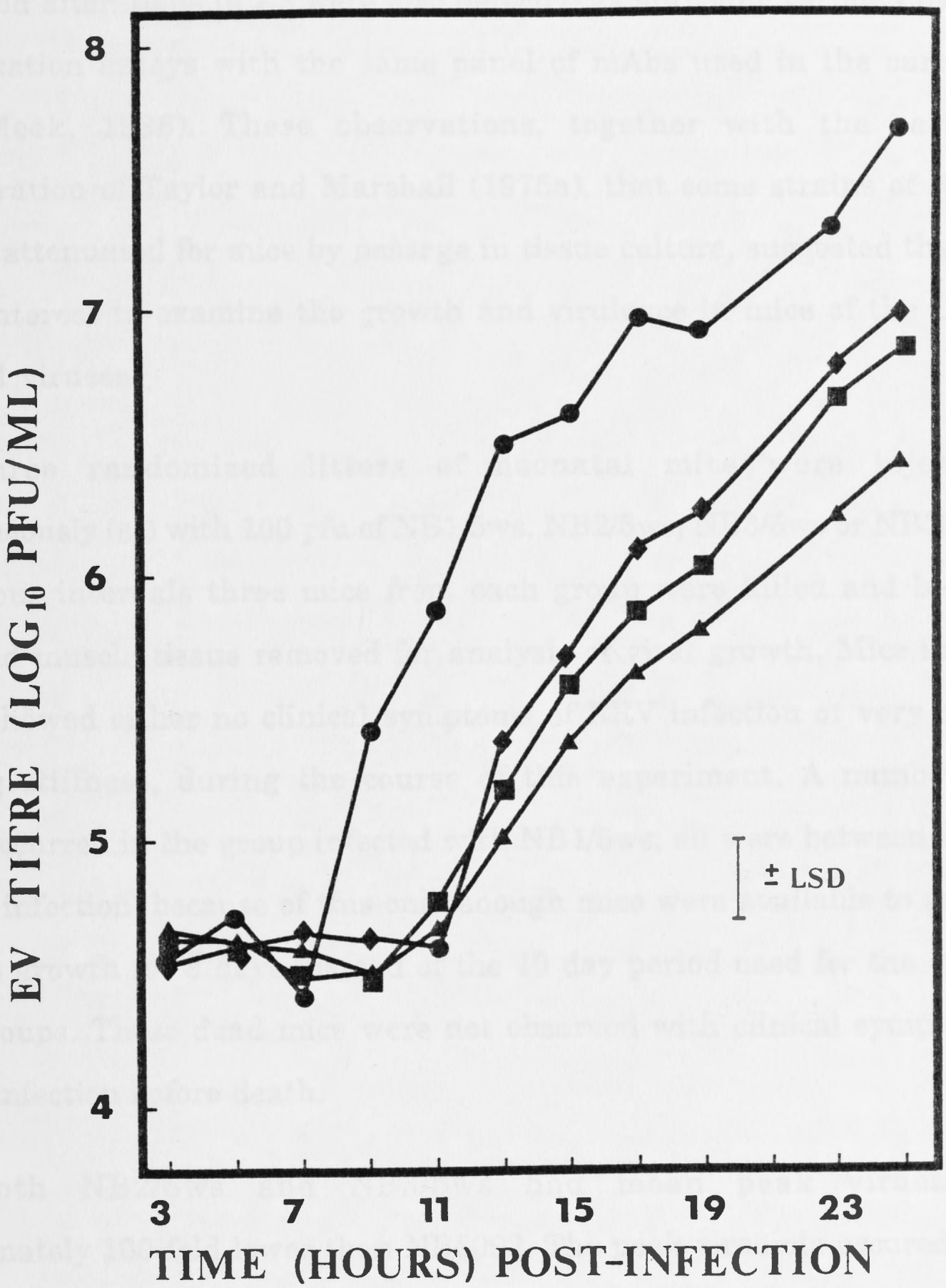
CEF monolayers in 35mm dishes were infected (moi~1) with RRV NB5092, NB1/5ws, NB2/5ws or NB3/5ws and incubated at 30°. Each virus was used to infect triplicate monolayers. Growth samples of the culture supernatants were taken at appropriate times and EV titres assayed by plaque formation on Vero cell monolayers at 36°.Statistical analysis was by analysis of variance. Error bars represent least significant difference.

NB5092 ●

NB1/5ws ▲

NB2/5ws ◆

NB3/5ws ■


8
7
6
5
4
EV TITRE (LOG10 PFU/ML)
± LSD
3
7
11
15
19
23
TIME (HOURS) POST-INFECTION

virulence although they may have enhanced it (Meek *et al.*,1989). Some amino acid alterations in E2 were also detected as antigenic variants using neutralization assays with the same panel of mAbs used in the current work (Meek, 1986). These observations, together with the earlier demonstration of Taylor and Marshall (1975a), that some strains of RRV could be attenuated for mice by passage in tissue culture, suggested that it was of interest to examine the growth and virulence in mice of the CEF passaged viruses.

Three randomized litters of neonatal mice were injected subcutaneously (sc) with 100 pfu of NB1/5ws, NB2/5ws, NB3/5ws or NB5092. At 24 hour intervals three mice from each group were killed and blood, brain and muscle tissue removed for analysis of viral growth. Mice in all groups showed either no clinical symptoms of RRV infection or very mild hind leg stiffness, during the course of this experiment. A number of deaths occurred in the group infected with NB1/5ws; all were between days 3-5 post infection, because of this only enough mice were available to assay for virus growth for 8 days instead of the 10 day period used for the other three groups. These dead mice were not observed with clinical symptoms of RRV infection before death.

Both NB2/5ws and NB3/5ws had mean peak viraemias approximately 100 fold lower than NB5092. The peak viraemia occured 3-4 days later and virus disappeared from the blood 2 days earlier in the course of infection with these two variants compared to NB5092. NB1/5ws also had a somewhat different pattern of viraemia to NB5092; peak levels occurred at day 5 for NB1/5ws compared to day 1 for NB5092 (Fig 4.8a). Virus titres in muscle followed the pattern set by the blood levels. NB2/5ws and NB3/5ws had substantially lower peak titres (10-100 fold) than NB5092 and virus disappeared from muscle tissue at around day 8-9, compared to NB5092 where virus was isolated from some mice at day 10. NB1/5ws had a

Figure 4.8

Titres of RRV NB5092 , NB1/5ws, NB2/5ws and NB3/5ws in the blood, muscle and brain of infected infant mice

Litters of neonatal mice were inoculated (sc) with 100 pfu of NB5092, NB1/5ws, NB2/5ws or NB3/5ws. At 24 hour intervals three mice from each group were killed; blood, hind leg muscle and brain were harvested. Virus titres were determined for each tissue, from each mouse, as pfu/g (wet weight) or pfu/ml, by plaque assay on Vero cell monolayers. Samples were collected until 10 days pi except for NB1/5ws where, due to the early deaths of several mice, samples were only collected up to day 8 pi. On day 10 only 2 mice infected with NB2/5ws or NB3/5ws were available.

Figure 4.8a

Titres of RRV NB5092, NB1/5ws, NB2/5ws and NB3/5ws in the blood of infected infant mice

Titres are the mean pfu/ml of the three mice assayed for each virus on each day, except as indicated above. Error bars represent least significant difference. Baseline titres are <500 pfu/ml, this is indicated by the arrow. All plaque assays were the mean of the number of plaques on duplicate monolayers. Where this was less than five plaques at the lowest dilution but some plaques were present, the titre was recorded as "trace". This is indicated by "t" on the graphs where a trace of virus was present in at least one mouse but the mean titre for all mice on that day was less than 500 pfu/ml.

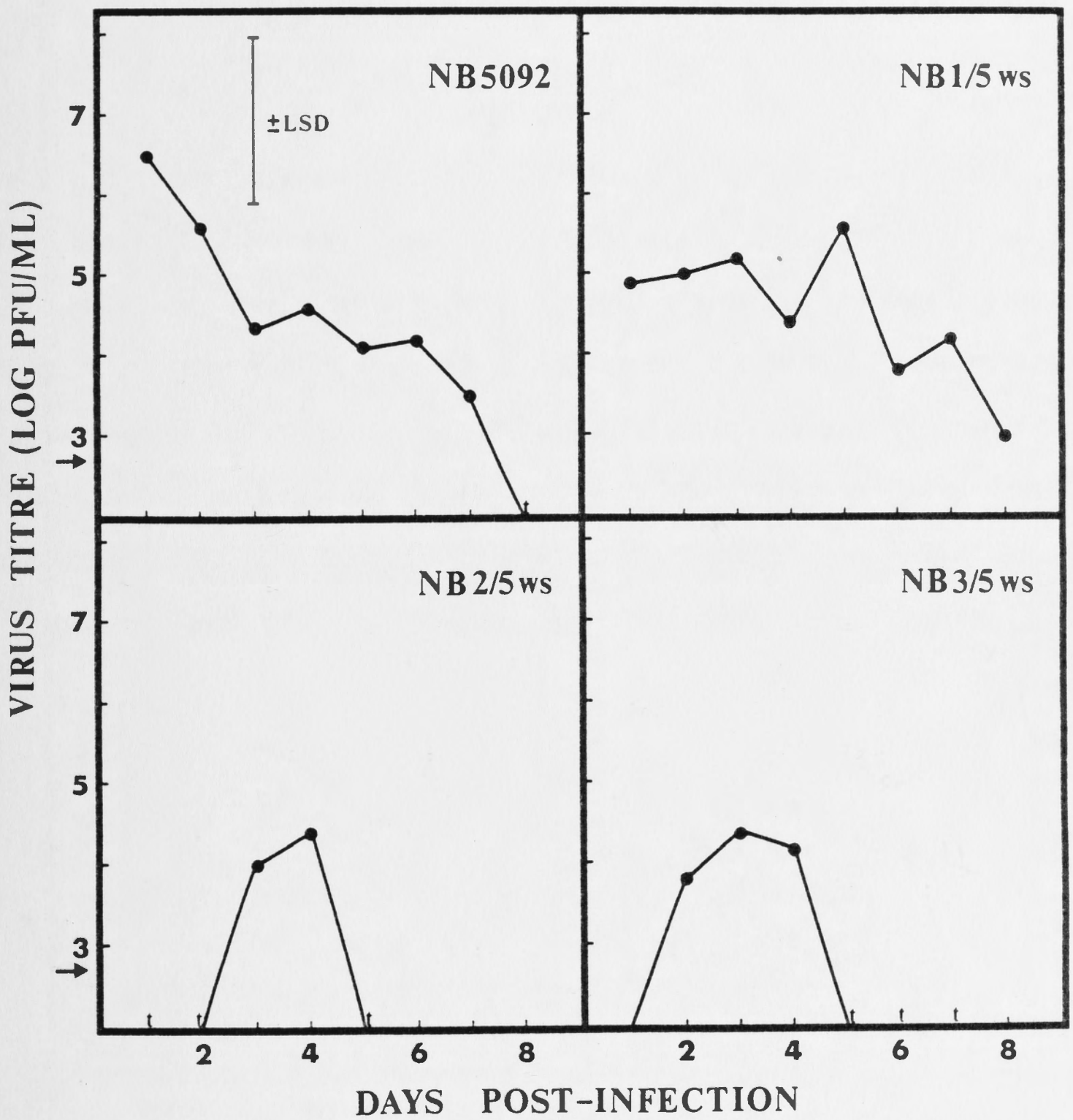

NB5092
±LSD
NB1/5 ws
NB2/5 ws
NB3/5 ws
VIRUS TITRE (LOG PFU/ML)
7
5
3
2
4
6
8
DAYS POST-INFECTION

Figure 4.8b

Titres of RRV NB5092, NB1/5ws, NB2/5ws and NB3/5ws in the muscle of infected infant mice

Titres are the mean pfu/g of the three mice assayed for each virus on each day, except as indicated above. Error bars represent least significant difference. Baseline titres are <500 pfu/g, this is indicated by the arrow. All plaque assays were the average of the number of plaques on duplicate monolayers. Where this was less than five plaques at the lowest dilution but some plaques were present, the titre was recorded as "trace". This is indicated by "t" on the graphs where a trace of virus was present in at least one mouse but the mean titre for all mice on that day was less than 500 pfu/g.

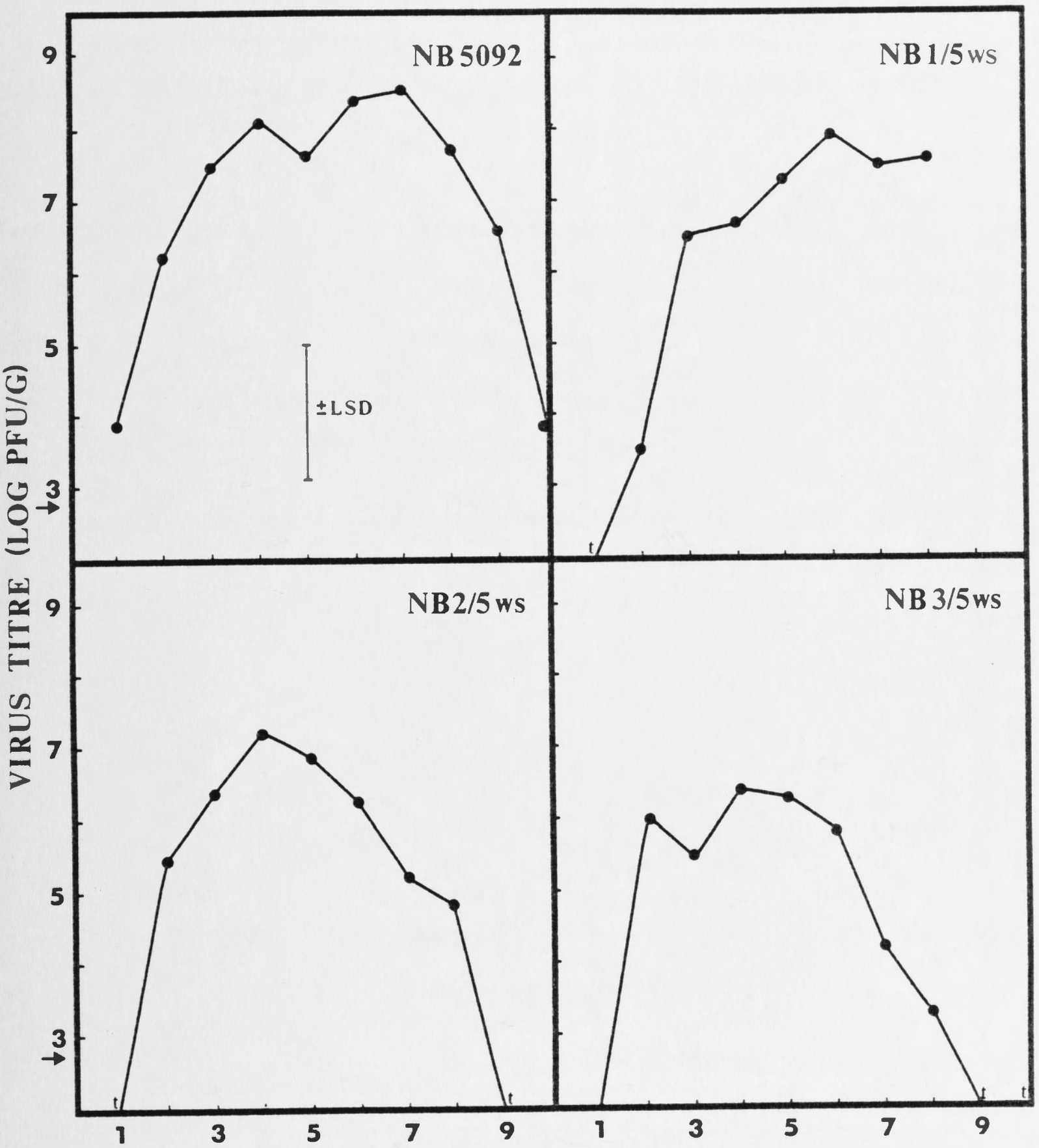
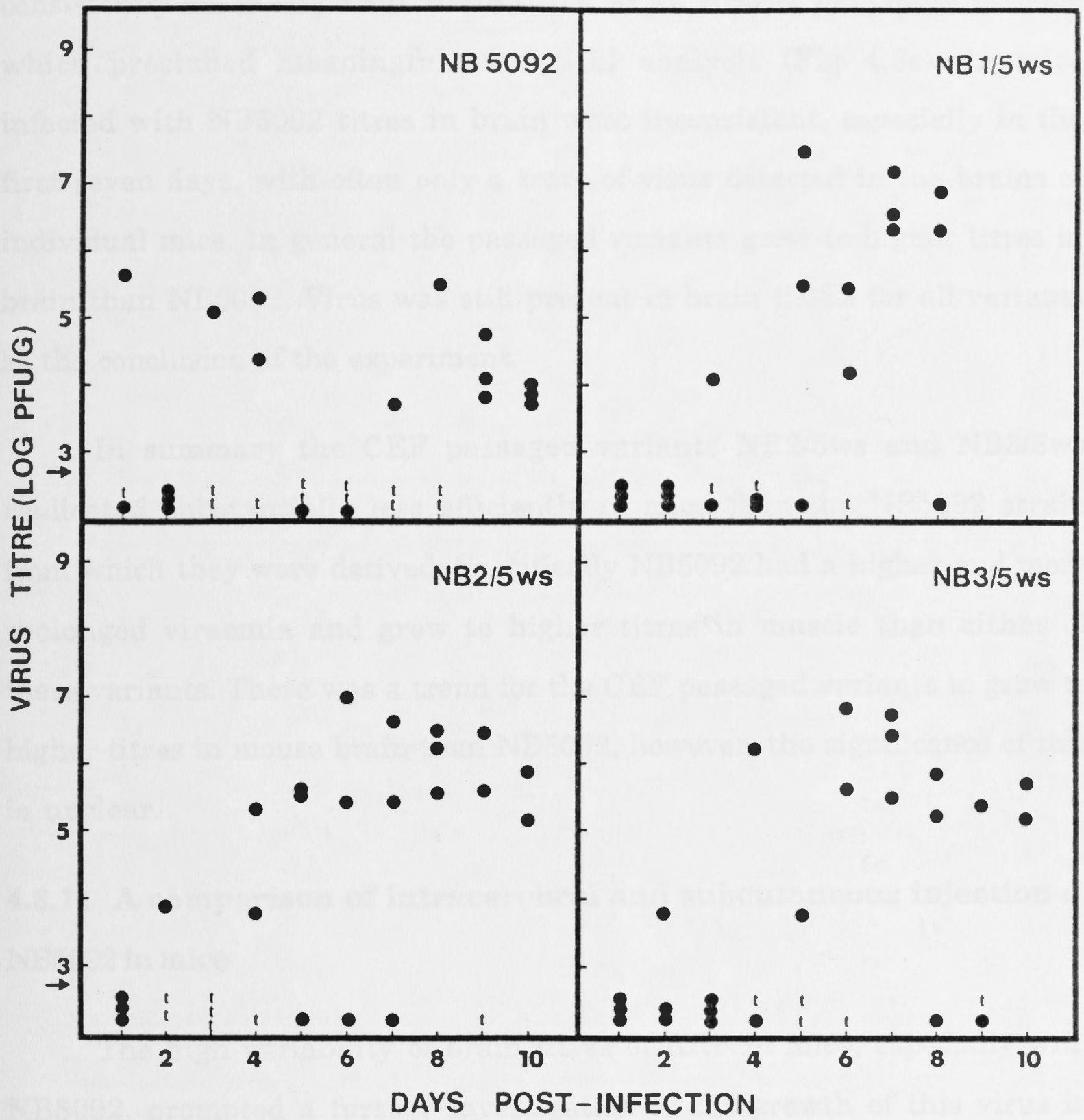

NB 5092
NB 1/5 ws
NB 2/5 ws
NB 3/5 ws
±LSD
VIRUS TITRE (LOG PFU/G)
9
7
5
3
1
3
5
7
9
DAYS POST-INFECTION

Figure 4.8c

Titres of RRV NB5092, NB1/5ws, NB2/5ws and NB3/5ws in the brains of infected infant mice

Brain titres for each mouse are shown as this data was too scattered for meaningful statistical analysis. Baseline titres are <500 pfu/g, this is indicated by the arrow. All plaque assays were the average of the number of plaques on duplicate monolayers. Where this was less than five plaques at the lowest dilution but some plaques were present, the titre was recorded as "trace". This is indicated by "t" on the graphs.


NB 5092
NB 1/5 ws
NB2/5ws
NB3/5ws
VIRUS TITRE (LOG PFU/G)
9
7
5
3
2
4
6
8
10
DAYS POST-INFECTION

similar pattern of muscle virus growth to NB5092 although titres were consistently lower (Fig 4.8b). Brain titres for all viruses were quite variable which precluded meaningful statistical analysis (Fig 4.8c). In mice infected with NB5092 titres in brain were inconsistent, especially in the first seven days, with often only a trace of virus detected in the brains of individual mice. In general the passaged variants grew to higher titres in brain than NB5092. Virus was still present in brain tissue for all variants at the conclusion of the experiment.

In summary the CEF passaged variants NB2/5ws and NB3/5ws replicated substantially less efficiently in mice than the NB5092 strain from which they were derived. Specifically NB5092 had a higher and more prolonged viraemia and grew to higher titres in muscle than either of these variants. There was a trend for the CEF passaged variants to grow to higher titres in mouse brain than NB5092, however, the significance of this is unclear.

4.3.11 A comparison of intracerebral and subcutaneous injection of NB5092 in mice

The high variability of brain titres of RRV in mice, especially with NB5092, prompted a further investigation of the growth of this virus in mouse brain. Meek *et al.* (1989) reported a similar pattern of growth of NB5092 in mouse brain but the individual variability appeared to be much less. In this experiment neonatal mice were injected either sc or intracerebrally (ic) with 100pfu of RRV. On days one to five pi brain and blood samples were collected, as previously described, from two mice in each group and assayed for virus.

The mean brain titre of NB5092 following sc injection was low for the 5 days post infection, reaching titres of 10^3-10^4 pfu/g. Intracerebral injection produced growth in brain to titres of 10^5-10^7 pfu/g (Table 4.5). This

Table 4.5

NB5092 growth in mice injected either subcutaneously or intracerebrally [a]

	Brain titre (log_{10} pfu/g)		Blood titre (log_{10} pfu/ml)	
	Route of inoculation [b]			
Day	sc	ic	sc	ic
1	<3	6.15	5.70	6.25
2	<3	5.57	4.30	6.04
3	4.0	6.45	3.70	5.48
4	3.18	6.32	3.84	5.08
5	3.40	7.32[c]	<3	4.64

[a]Neonatal mice were injected subcutaneously or intracerebrally with 100 pfu of NB5092. Blood and brain were collected from two mice from each group on days 1-5 post-infection. Virus titres in each tissue were determined in pfu/ml or pfu/g (wet weight) on Vero cell monolayers and the mean titres are shown for each day.

[b] sc, subcutaneous injection; ic, intracerebral injection.

[c] on day 5 only one mouse was available from the ic group.

difference would be expected if the block in brain replication was due to interference in brain entry rather than an inability to replicate in brain tissue. This experiment provided further corroboration that the assay technique used was adequate to detect virus in the brain as high titres were found in the ic injected mice while the sc injected mice had similar brain titres to those found in the previous experiment.

Two further observations are of interest: (1) brain titres in the sc infected group did not start to rise until after day two which is comparable with those of the CEF passaged clones in mouse brain (see Fig 4.8c). (2) Blood titres for NB5092 can be quite variable but the same pattern of highest titre on day 1, that was observed in the mouse growth assays (Fig 4.8a) was also seen in both the ic and sc infected groups in this experiment.

4.3.12 Virulence assay of NB1/5ws and NB5092 in mice

A number of observations suggested that NB1/5ws may have been of higher virulence for mice than NB5092. These included the death of several mice during growth assays and the higher brain titres observed in some mice infected with NB1/5ws compared with mice infected with NB5092. Meek *et al.* (1989) noted that increased growth of NB5092 variants in mouse brain was correlated with increased virulence. Therefore the virulence of NB5092 and NB1/5ws was compared in neonatal mice.

Litters of 10-12 neonatal mice were injected sc with 10^2, 10^3, 10^4 or 10^5 pfu of each virus and observed daily for clinical symptoms. No deaths occurred in either treatment group. For NB5092 clinical symptoms of mild/moderate hind leg weakness were observed in all four dose groups between days 8 and 13. Signs of severe hindleg paralysis were observed in 1 of 10 mice in each of the two highest dose groups. All mice were clinically normal by day 14. For NB1/5ws mild symptoms of hind leg stiffness were seen in all groups from day 8-12. No severe clinical symptoms were

observed and all mice recovered uneventfully by day 14. It was concluded that NB1/5ws was not more virulent than NB5092 and may be slightly less virulent, as the clinical effect of infection appeared somewhat milder. Therefore the deaths in the previous experiment were presumably due to factors other than the injected virus and may have related to mismothering or some other form of stress.

4.3.13 Antigenic analysis of virus recovered from mouse muscle

To examine whether antigenic revertants were selected during virus growth in mice, virus isolated from muscle from the day of peak muscle titre was examined using a PRNA against mAbs NB3C4 and T10C9. Working stocks of each virus were assayed in parallel as controls (Table 4.6). The populations assayed showed no evidence of antigenic reversion at the epitopes characterized by these mAbs.

4.3.14 Nucleotide sequencing studies of CEF passaged variants of NB5092

The results presented above show that two classes of antigenic variants were isolated after passaging RRV NB5092 in avian cells. These antigenic variants did not revert on backpassaging in BHK cells and were associated with alterations of virus growth in mice. Nucleotide sequencing of the complete E1 and E2 genes of the antigenic variants was performed to determine the changes in structural glycoproteins associated with passaging. Sequence data was obtained for NB1/5ws, NB2/5ws and NB3/5ws using IC/RNA from either CEFs or BHK cells. NB5092 IC/RNA (extracted from BHK cells) was sequenced in parallel with the passage variants. The dideoxy chain termination method (Sanger *et al.*,1977) using extension from oligonucleotide primers was used in all studies (Methods; 4.2.9).

Table 4.6

Antigenic analysis of virus isolated from muscle of mice infected with NB5092, NB1/5ws, NB2/5ws or NB3/5ws

	mAb neutralization titre [a]	
Virus [b]	**NB3C4**	**T10C9**
NB5092	5.71	5.53
NB5092 (m)	5.50	5.66
NB1/5ws	3.98	nd
NB1/5 (m)	3.88	nd
NB2/5ws	nd	2.58
NB2/5 (m)	nd	3.06
NB3/5ws	nd	2.48
NB3/5 (m)	nd	2.63

[a] The neutralization titres, with either mAb NB3C4 or mAb T10C9, of virus isolated from infected mouse muscle and the stock used for infection, are shown as the $\log_{10}$ mAb dilution producing 50% neutralization in PRNA. ten fold dilution intervals were used. Differences in titre of greater than 10 fold were regarded as significant.

[b] Mouse muscle virus is indicated by (m) after the virus code. Mouse muscle virus was from the day of peak titre as follows; NB5092(m)=mouse 1, day 6; NB1/5(m)=mouse3 day 7; NB2/5(m)=mouse2 day 5; NB3/5(m)=mouse 1 day 5.

nd, not tested

The E2 gene runs from nucleotide 1,048 in the NB5092 26S RNA to nucleotide 2,313 and encodes 422 amino acids in 1,266 nucleotides. The E1 gene extends from nucleotide 2,494 to nucleotide 3,807 and encodes 438 amino acids in its 1,314 nucleotides (Faragher *et al.*,1988). A single G→A transition was identified in the E2 gene of NB1/5ws at position 1,057 leading to a deduced amino acid alteration of Glu→Lys at amino acid 4 of the E2 glycoprotein. No other nucleotide changes were identified in either E1 or E2 for this variant, however, sequence was not obtained for 55 nucleotides in E1 (Methods; 4.2.9). The variants NB2/5ws and NB3/5ws both had a single C→A transversion in the E2 gene at nucleotide 1,701, leading to an amino acid change of Asn→Lys at position 218 in the E2 glycoprotein. No other nucleotide alterations were detected (Table 4.7). Thus all three variants had a change to a positively charged amino acid in E2. Amino acid 218 is close to epitope a whereas position 4 is not near any known neutralization epitope in the primary sequence.

4.3.15 Location of amino acid alterations in the E2 glycoprotein of CEF passaged RRV in relationship to conserved amino acid sequence, hydropathy profile and predicted secondary structure

To obtain further information about the possible significance of the amino acid changes at position 4 and 218 of E2 the amino acid sequence of NB5092 E2 was compared with the aligned sequence for RRV T48, SFV and SIN. Hydropathy profiles, using the Kyte and Doolittle (1982) algorithm, and secondary structure predictions using the methods of Chou and Fasman (1978) and Garnier *et al.* (1978) were generated for NB5092 and the CEF passaged variants using the PeptideStructure program and presented graphically using PlotStructure (Jameson and Wolf, 1988; Wolfe *et al.*,1988).

Table 4.7

Nucleotide changes and deduced amino acid changes in CEF passaged variants

Variant	nucleotide change and number [a]	amino acid change and number
NB1/5ws	GAG→AAG (1057)	Glu(4)→Lys
NB2/5ws	AAC→AAA (1701)	Asn(218)→Lys
NB3/5ws	AAC→AAA (1701)	Asn(218)→Lys

[a] Nucleotides are numbered from the 5' end of the NB5092 26S RNA; amino acids are numbered from the N-terminus of E2 (Faragher *et al.*,1988).

The alteration at amino acid 218 in E2 of CEF passaged NB5092 is in a hydrophilic region of the protein as predicted by the Kyte and Doolittle (1982) algorithm. The hydropathy profile is not strongly conserved in the region surrounding amino acid 218 between RRV NB5092, T48, SFV and SIN (Fig 4.9). Amino acid 218 is in the middle of a five amino acid motif; Thr-Ile-Asn-Thr-Cys, which is completely conserved between NB5092, T48 and SFV (Fig 4.10). The substitution of a charged lysine residue for the uncharged asparagine, could increase the hydophilicity of this region which is adjacent to epitope a, at position 216, and is within the major neutralization domain previously identified on E2 (Vrati *et al.*,1988). Amino acid 4 is at the beginning of a strongly conserved region of amino acid sequence for the alphaviruses RRV, SFV and, to a lesser extent, SIN (Fig 4.10). The N-terminus of NB5092 is predicted to be hydrophobic because of an Ile residue at position 3 and thus differs from RRV T48 which has a Thr at this position and a predicted hydrophilic N-terminus (Fig 4.9). Secondary structure predictions are of limited accuracy with a predictive value for any residue of approximately 66%, this is improved where two methods predict similar structures (Schulze and Schirmer, 1979). In this case secondary structure analysis predicted that the Glu→Lys change at amino acid 4 altered a region of alpha helix to β–sheet while that at 218 is in a region of predicted reverse turns and random coil structure (Fig 4.10).

The location of the amino acid 218 change within a hydrophilic region of the E2 glycoprotein together with the change in antigenic reactivity strongly suggests that this residue is on the surface of the protein and that the predicted change, involving an alteration in charge, could alter the surface conformation of the E2 glycoprotein and its interactions with other molecules. Amino acid 4 is predicted to be adjacent to a hydrophobic region caused by the need to bury the hydrophobic side chain of the Ile at position 3. The change in NB1/5ws charge together with the

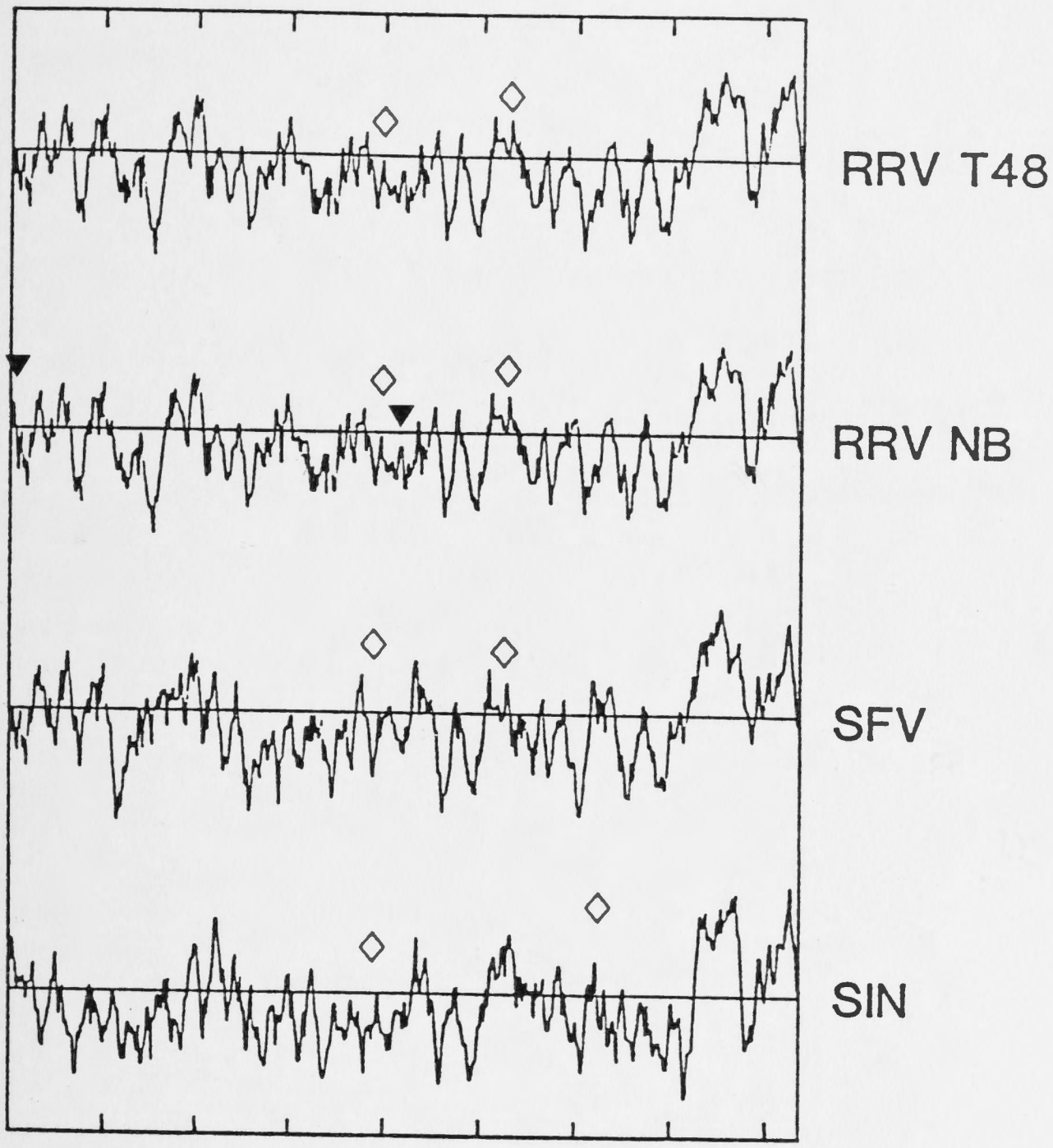
Figure 4.9

Hydropathy profiles of the E2 protein of RRV NB5092, RRV T48, SFV and SIN*

The hydropathy profile of E2 of RRV NB5092, RRV T48, SFV and SIN was determined using the algorithm of Kyte and Doolittle (1982). Hydrophobicity increases with distance above the horizontal line, hydrophilicity increases with distance below the horizontal line. The location of amino acid changes in CEF passaged variants (▼) and of glycosylation sites (◆) are indicated. Tick marks on the baseline represent 50 amino acid intervals. Search length was seven amino acids.

Sequence data was from the following sources: RRV T48, Dalgarno *et al.* (1983); NB5092, Meek (1986); SFV, Garoff *et al.* (1980); SIN, Rice and Strauss (1982).

* adapted from Meek (1986).


RRV T48
RRV NB
SFV
SIN

```
NB  E Y Q W G N N P P V R L W A Q L T T E G K P H G W P H E I I  E2 (355)
T48 . . . . . . . . . . . . . . . . . . . . . . . . . . . . .     (355)
SFV . . H . . . . D . . . . . S . . . . . . . . . . . . Q . V     (355)
SIN . . I . . . H E . . . V Y . . E S A P . D . . . . . . . V     (356)

NB  Q Y Y Y G L Y P A A T I A A V S G A S L M A L L T L A A T C  E2 (385)
T48 . . . . . . . . . . . . . . . . . . . . . . . . . . . . .     (385)
SFV . . . . . . . . . . . V S . . V . M . . L . . I S I F . S C     (385)
SIN . H . . H R H . V Y . . L . . A S . T V A M M I G V T V A V     (386)

NB  C M L A T A R R K C L T P Y A L T P G A V V P L T L G L L X  E2 (415)
T48 . . . . . . . . . . . . . . . . . . . . . . . . . . . . C     (415)
SFV Y . . V A . . S . . . . . . . . . . . A . . W . . . I . C     (415)
SIN L C A C K . . . E . . . . . . . A . N . . I . T S . A . . C     (416)

NB  C A P R A N A                                              E2 (422)
T48 . . . . . . .                                                 (422)
SFV . . . . . H .                                                 (422)
SIN . V R S . . .                                                 (423)
```

A colisting of the amino acid sequence of E2 for RRV NB5092, RRV T48, SFV and SIN. Dots indicate that the residue is identical to the NB5092 sequence, gaps have been introduced to maximize homology. Potential glycosylation sites are underlined. The positions of differences between NB5092 and the CEF passaged variants are indicated (▼). Sequence data is from Dalgarno *et al.* (1983) and Faragher *et al.* (1988).

Figure 4.10

Colisting of RRV NB5092, T48, SFVand SIN E2 amino acid sequences

```
                    ▼
NB  S V I E H F N V Y K A T R P Y L A X C A D C G D G Y F C Y S  E2 (30)
T48 . . T . . . . . . . . . . . . . . Y . . . . . . . . . . .     (30)
SFV . . S Q . . . . . . . . . . . . I . . . . . . A . H S . H .   (30)
SIN . . . D G . T       L . S . . . G T . S Y . H H T E P . F .   (27)

NB  P V A I E K I R D E A S D G M L K I Q V S A Q I G L D K A G  E2 (60)
T48 . . . . . . . . . . P . . . . . . . . . . . . . . . . . .     (60)
SFV . . . . . A V . S . . T . . . . . . . F . . . . . I . . S D   (60)
SIN . . K . . Q V W . . . D . N T I R . Q T . . . F . Y . Q S .   (57)

NB  T H A H T K M R Y M       A G H D V Q E S K R D S L R V Y T  E2 (87)
T48 . . . . . . I . . .       . . . . . . . . . . . . . . . .     (87)
SFV N . D Y . . I . . A       D . . A I E N A V . S . . K . A .   (87)
SIN A A S A N . Y . . . S L K Q D . T . K . G T M . D I K I S .   (87)

NB  S A A C S I H G T M G H F I V A H C P P G D Y L K X S F E D  E2 (117)
T48 . . . . . . . . . . . . . . . . . . . . . . . V . . . .     (117)
SFV . G D . F V . . . . . . . . L . K . . . . E F . Q V . I Q .   (117)
SIN . G P . A A L S Y K . Y . L . . K . . . . . S V T V . I V S   (117)

NB  A N S H V K A C K V Q Y K H D P L P V G R E K F V V R P H F  E2 (147)
T48 . D . . . . . . . . . . . . . . . . . . . . . . . . . . .     (147)
SFV T R N A . R . . R I . . H . . . Q . . . . . . . T I . . . Y   (147)
SIN S   N S A T S . T L A R . I K . K F . . . . . Y D L P . V H   (146)

NB  G V E L P C T S Y Q L T T A P T D E E I D M H T P P D I P D  E2 (177)
T48 . . . . . . . . . . . . . . . . . . . . . . . . . . . .     (177)
SFV . K . I . . . T . . Q . . . E . V . . . . . . M . . . T . .   (177)
SIN . K K I . . . V . D R L K E T . A G Y . T . . R . R P H A Y   (176)

NB  R T L L S Q T A G N V   K I T A G G R T I R Y N C T C G R D  E2 (206)
T48 . . . . . . . . . . .   . . . . . . . . . . . . . . . .     (206)
SFV . . . . . Q S . . .     . . . V . . K.K.V K . . . . . . T G   (206)
SIN T S Y . E E S S . K . Y A K P P S . . N . T . E . K . . D Y   (206)

                    ▼
NB  N V G T T S T D K T I N T C   K I D Q C H A A V T S H D K W  E2 (235)
T48 . . . . . . . . . . . . . .   . . . . . . . . . . . . . .     (235)
SFV . . . . . N S . M . . . . .   L . E . . . V S . . D . K . .   (235)
SIN K T . . V . . R T E . T G . T A . K . . V . Y K S D Q T . .   (236)

NB  X F T S P F V P R A D Q T A R K G K V H V P F P L T N V T C  E2 (265)
T48 Q . . . . . . . . . . . . . R . . . . . . . . . . . . .     (265)
SFV Q . N . . . . . . . . E P . . . . . . . I . . . . D . I . .   (265)
SIN V . N . . D L I . H . D H T A Q . . L . L . . K . I P S . .   (266)

NB  R V P L A R A P D V T Y G K K E V T L R L H P D H P T X F S  E2 (295)
T48 . . . . . . . . . . . . . . . . . . . . . . . . . L . .     (295)
SFV . . . M . . E . T . I H . . R . . . . H . . . . . . . L . .   (295)
SIN M . . V . H . . N . I H . F . H I S . Q . D T . . L . L L T   (296)

NB  Y R S L G A V P H P Y E E W V D K F S E R I I P V T E E G I  E2 (325)
T48 . . . . . . E . . . . . . . . . . . . . . . . . . . . .     (325)
SFV . . T . . E D . Q Y H . . . . T A A V . . T . . . P V D . M   (325)
SIN T . R . . . N . E . T T . . I V G K T V . N F T . D R D . L   (326)
```

Epitope [1], defined by mAb NIH-C4, has been suggested to involve amino acids 232 and 234 of E2 by selection of escape mutants (Wahl et al. 1987). A deletion of amino acids 234-241 in E2 of RRV-T48 also reduces neutralization by this mAb (Wahl et al. 1989a). The glycosylation of a NXT site, at amino acid 6, affecting neutralization by mAb WNE-4 suggests that the E2

change in mAb reactivity and the predicted change in secondary structure suggest that the mutation at this site possibly alters local protein conformation.

4.4 Discussion

Passaging in avian cells selected variants of RRV NB5092 which had single amino acid changes in the E2 glycoprotein. Two types of variant were found; one, changed at amino acid 4 (Glu→Lys) and the other at 218 (Asn→Lys). Both classes of variant were defined by changes in neutralization by mAbs. Variants with the Asn218→Lys difference were less efficiently neutralized by mAb T10C9 than the parental NB5092. The Glu4→Lys alteration in NB1/5ws produced less efficient neutralization by mAbs NB3C4 and T1D11. The two variants with the alteration at 218 (NB2/5ws and NB3/5ws) were both characterized by attenuated growth in mice, as measured by virus levels in blood and muscle.

4.4.1 Antigenic changes in E2 of CEF selected variants

Monoclonal antibody T10C9 defines epitope a at amino acid 216 on E2 (Vrati *et al.*,1988). This is within the region between 216-251 identified as containing a major neutralization determinant of RRV. The data presented in this chapter demonstrated that an alteration in 218 from a neutral to a positively charged amino acid is sufficient to inhibit, but not abolish, neutralization by this mAb.

Epitope b1, defined by mAb NB3C4, has been mapped to amino acids 232 and 234 of E2 by selection of escape mutants (Vrati *et al.*,1988). A deletion of amino acids 55-61 in E2 of RRV T48 also reduces neutralization by this mAb (Vrati *et al.*,1986). The demonstration of a third site, at amino acid 4, affecting neutralization by mAb NB3C4 suggests that the N-

terminal region of the E2 protein is folded into apposition with epitope b1. This is supported by the results with mAb T1D11 which defines epitope c (Vrati *et al.*,1988). This epitope has not been mapped by escape mutants as it has not proved possible to select resistant variants *in vitro*. NB5092 is neutralized less efficiently by this mAb than T48 (P.J. Kerr unpublished) and in competitive binding assays (CBAs) it competes with mAbs NB3C4 and T1E7 which define epitopes b1 at 232-234 and b2 at 246-251 on E2 (Vrati *et al.*,1988). Neutralization of NB5092 by T1D11 is almost abolished by the Glu4→Lys change in NB1/5ws which suggests this amino acid is very close to the binding site of T1D11. Although the possibility that the amino acid change is affecting a binding site at a distance cannot formally be ruled out (Blondel *et al.*,1986).

4.4.2 Attenuation in mice of the CEF selected variants NB2/5ws and NB3/5ws

The two variants changed at E2 218 had similar patterns of mouse growth characterized by a low level of viraemia of short duration and reduced growth in muscle compared to NB5092. Two independently selected variants with the Asn218→Lys change in E2 had virtually the same mouse growth characteristics, while a third variant selected in parallel but with a change at amino acid 4 had a different pattern of mouse growth that more closely resembled that of NB5092. This is consistent with amino acid 218 being involved in modulating RRV NB5092 replication in mice, possibly by altering tissue tropism. It is not possible to exclude changes in nonstructural proteins or untranslated regions from involvement in this attenuation. This would require isolating clones without the change at 218 and assaying their growth in mice. Meek *et al.* (1989) showed that amino acid changes in E2 were selected during passaging RRV NB5092 in mice. Changes at positions 212, 232 and 251 were close to or within defined neutralization epitopes. It was proposed

that alterations in E2 were involved in adaptation to mouse cells. It is interesting to note that the only amino acid change found in E2 during the course of the RRV epidemic in the Pacific Islands, where humans appear to have been the principal vertebrate host, was at residue 219; Thr→Ala (Burness *et al.*,1988). However, this did not result in changes in neutralization by the mAbs used here (Burness *et al.*,1988).

A similar interaction between single amino acid alterations in neutralization epitopes, tissue tropism and virulence is seen in other viruses. For SIN, as already discussed in Chapter 3, selection for rapid penetration in tissue culture leads to changes in mAb reactivity and alterations in mouse virulence. These phenotypic changes are due to an alteration at amino acid 114 or to the addition of a glycosylation site at amino acid one of E2 which prevents PE2 cleavage (Olmsted *et al.*,1986; Davis *et al.*,1986; Polo *et al.*,1988; Russell *et al.*,1989). For influenza virus, passaging in avian or mammalian cells or selection of mAb resistant variants can lead to changes in tissue tropism or receptor recognition which appear to be generated by single amino acid alterations, in the haemagglutinin protein, adjacent to the receptor binding site (Philpott *et al.*,1990; Robertson *et al.*,1985; Deom *et al.*,1986; Rogers *et al.*,1983). Selection of mAb resistant variants produced similar effects on tissue tropism and virulence for other viruses, for example reovirus (Spriggs and Fields, 1982) and rabies (Tuffereau *et al.*,1989). For human immunodefficiency virus a single amino acid alteration in the gp120 protein abrogates receptor binding, while other amino acid changes in the same region alter tissue tropism (Cordonnier *et al.*,1989).

4.4.3 Mechanism of selection of NB5092 antigenic variants in CEFs

It is highly probable that the amino acid changes in E2 have been specifically selected during passaging in avian cells, for the following

reasons. Starting from three independently cloned stocks, the number of passages was very small providing little opportunity for the accumulation of neutral mutations. In support of this no silent nucleotide changes were found in some 7.5 kb of nucleotide sequence analysis, covering the E1 and E2 genes from three independently passaged and plaque purified virus populations. For comparison the NB5092 strain of RRV differs from the T48 strain at 284 nucleotides; 81% of nucleotide differences in the coding regions are silent (Faragher *et al.*,1988). In the E2 gene, 5 of 32 changes are coding while in E1 only 3 out of 30 changes are coding.

By passaging a virus in tissue culture it is possible to select variants with genetic and phenotypic alterations. This raises the question of whether such alterations relate to adaptation to the new cell type or are a response to selection pressure generated by the process of passaging itself, for example, generation of interfering activity, different temperature conditions (perhaps relaxing constraints on protein conformation), or different composition of culture medium.

The mechanism of selection of the antigenic variants in CEFs is unclear. The simplest hypothesis is that the antigenic variants were able to outgrow the parental NB5092. Limited experimental data indicated that for NB1/5, non-plaque purified virus had a shorter lag period in CEFs than NB5092, supporting the idea of more rapid growth in chick cells. However, detailed analysis of extracellular virus production, using the plaque purified NB1/5ws, NB2/5ws and NB3/5ws showed that all three had a substantially longer lag period in CEFs than NB5092. Domingo *et al.* (1978) showed in competition experiments with bacteriophage Qβ that the faster growing wild-type outgrew mutants which had a relative replication rate of 0.8-0.9. Thus it appears that effects on growth rates under the conditions of passaging must be different to those in the growth assays to allow the

slower growing antigenic variants to become a substantial proportion of the population.

It is possible that the increased positive charge on the variants is associated with an enhanced affinity for chick cells which in some circumstances gave the variant an advantage over NB5092. Analysis of RNA synthesis kinetics would provide further information on the lag period of viral replication. It is also possible that there are nonstructural protein changes or mutations in regulatory regions which were involved in selection in CEFs. Further experimentation along the lines indicated would be required to understand the mechanism by which selection has proceeded.

All three passage series had a dramatic decrease in extracellular virus titre from the second passage with recovery on fifth passage. Antigenic analysis of plaques picked from intermediate passage levels indicated that the NB1/5ws and NB2/5ws types were a major proportion of the passaged population after two to three passes. This was confirmed by antigenic analysis of the fifth passage non plaque purified populations. However, in passage series three the selected NB3/5ws antigenic variant did not become predominant. This suggests that selection of the analysed variants had little to do with the increase in titre on the fifth passage. It is possible that the major selection occurred during the first and second passages and that titre changes were related to extrinsic factors in the passaging.

It appears that some form of interfering activity developed in the early stages of passaging which inhibited viral replication and that this was either selected against or diluted out during passaging. Experiments in which virus at various passage levels was grown in the presence or absence of AMD indicated no advantage to this treatment suggesting that

interferon was not responsible. The drop in titre followed by recovery and the very low levels of RNA synthesis observed when first passage virus was used to infect CEFs are suggestive of defective interfering (DI) particles (Holland *et al.*,1982). Martin *et al.* (1979) demonstrated DI particle RNA and interfering activity in RRV passaged in BHK cells. This was associated with higher rates of RNA synthesis compared to the unpassaged controls although both RNA synthesis and virus growth lagged behind that of the unpassaged virus. Defective interfering activity has been reported in a number of viral systems (Holland *et al.*,1982) usually associated with high moi passaging or persistent infections. In the case of RRV in CEFs, if DI particles were to play a role they would have to accumulate very early in the passage series, it is possible that passaging in an avian cell line at 30° encourages this. The demonstrated heterogenous nature of the CEF population may also play a role. Similar drops in titre have been described in yellow fever virus passaged in HeLa cells (Hardy, 1963; Converse *et al.*,1971; Dunster *et al.*,1990).

4.4.4 The temperature sensitive replication of RRV in CEFs

Work with RNA synthesis kinetics and virus growth at 36° in CEFs showed a failure of detectable RNA synthesis. NB5092 but not T48 replicated efficiently in CEFs at 30°. This, combined with temperature shift experiments, suggests that the ts block is at the post-attachment stage, involving cell entry and uncoating or RNA synthesis. The genetic differences between RRV T48 and NB5092 (Faragher *et al.*,1988) are too great for further molecular dissection of this difference between the viruses. An additional complication in investigating this temperature sensitive block was that growth of NB5092 was quite variable in secondary CEFs at 36°, this probably reflected the heterogenous nature of the cell population. It is possible that the lower temperature requirement is one

reason for the poor replication of RRV in birds which typically have higher body temperatures than mammals.

One aspect that has not been considered in this discussion until now because of its speculative nature, is that the passage of NB5092 in CEFs at 30° was also selecting for virus, with ts or other mutations, which did not plaque on Vero cell monolayers at the standard temperature of 36° and that this then resulted in an apparently decreased titre. This hypothesis would require that such virus was then outgrown by the antigenic variants or other variants, which would appear dominant in the population at early passage levels because other virus types did not form plaques. No attempt was made in this work to look for ts mutations, however, there was no evidence that the antigenic variants characterized had any growth disadvantage in mammalian cells at 36° based on the titres of the variants when grown in BHK cells.

4.4.5 Conclusion

In summary, passaging RRV NB5092 in CEFs selected variants with amino acid alterations in the E2 protein at either position 4 or 218. Both changes involved selection of a basic lysine residue. The mechanism of selection is unclear but three lines of evidence suggest that these changes occurred in functionally important regions of the protein. Firstly both amino acid changes altered neutralization by one or more mAbs indicating that the conformation of neutralization epitopes was altered. As already discussed neutralization epitopes are likely to be adjacent to, or part of, sites on the protein involved in virus replication. Secondly no other changes, either silent or coding were found in the nucleotide sequence of E1 or E2 of the passage variants suggesting that there had been positive selection pressure for the changes found. Thirdly the amino acid alterations altered the growth characteristics of NB5092 in mice. This was

most obvious for the change at 218 which was associated with a lowered viraemia and decreased muscle titre.

Chapter 5

The effect of growth and passaging in human or arthropod cells on the E2 glycoprotein of RRV

5.1 Introduction

In Chapter 4 the selection and characterization of passage variants of RRV NB5092 in avian cells was examined. These variants had been selected during passage in CEFs and were defined by antigenic and genetic changes in the E2 glycoprotein. RRV does not normally replicate in birds, therefore these changes may represent specific adaptation to avian cells. It was of interest to define changes in E2 selected on passaging RRV in other cell lines, examine the relationship of such changes to those that were selected in avian cells and to define whether different changes were selected in different strains of RRV. As discussed in chapter 4, such changes could be important in defining regions associated with virus-cell interactions. Therefore two strains of RRV (T48 and NB5092) were passaged in human and mosquito cells.

Humans are frequently regarded as an incidental host for alphaviruses rather than being involved in transmitting virus to mosquitoes (Chamberlain, 1980). However, during the epidemic of polyarthritis in the Pacific Islands humans appeared to be the predominant vertebrate host for RRV and, in contrast to previous epidemics within Australia, the virus was isolated routinely from people with clinical symptoms (Aaskov *et al.*,1981a; Rosen *et al.*,1981; Tesh *et al.*,1981; Marshall and Miles, 1984; see Chapter 1). During the course of this epidemic only one nucleotide alteration was found in E2. This led to an amino acid alteration at position 219 (Burness *et al.*,1988) which is very close to epitope a at 216 and to 218, at which changes were selected on passaging in CEFs.

For alphaviruses, Durbin and Stollar (1986) demonstrated that passaging a strain of SIN in mosquito cells selected for amino acid alterations in E2 one of which was associated with host cell restriction. In

another group of arboviruses, the Bunyaviruses, neutralization of La Crosse virus by some mAbs depended on whether the virus was assayed in mosquito or vertebrate cells suggesting that the mAb epitope is neutralizing in one cell type but not the other. One epitope was only neutralizing in mosquito cells and it was suggested that this may represent an important determinant in the interaction of Bunyaviruses with insect cells (Grady and Kinch,1985). In addition a mAb resistant variant of LaCrosse virus was restricted in its ability to infect mosquitoes (Sundin *et al.*,1987). Stable genetic changes accumulating in the surface glycoproteins on passaging in mosquito cells also altered neutralization of Bunyaviruses by a panel of mAbs (James and Millican, 1986). It is possible that these epitopes are important determinants of cell specificity in Bunyaviruses and that similar determinants could be defined for RRV.

A further aspect of alphavirus biology is the change in glycoprotein structure caused by host cell modification. This is exemplified by replication in mosquitoes. Mosquito cells do not add complex oligosaccharide chains containing sialic acid to E1 and E2, but only add high mannose carbohydrate moieties (Stollar *et al.*,1976; Luukkonen *et al.*,1977; Hsieh and Robbins, 1984). Differences in the size and charge of carbohydrate moieties could change the antigenic reactivity of virus grown in mosquito cells compared to vertebrate cell grown virus. For SIN, Stollar *et al.* (1976) did not demonstrate any differences in antigenicity, using polyclonal antisera, between virus grown in mosquito, avian or mammalian cells. However, there is no data on antigenic changes involving mAb epitopes.

The RRV mAbs, T1E7, T10C9 and NB3C4 have been mapped to epitopes close to the glycosylation sites at asparagine 200 and 262 on the RRV E2 glycoprotein (Vrati *et al.*,1988; see Chapter 2). By analogy with Sindbis virus (Mayne *et al.*,1985) the first of these sites is expected to have a

complex oligosaccharide. Therefore it was of interest to examine whether RRV strains grown in mosquito cells and therefore phenotypically altered in carbohydrate and charge at complex glycosylation sites were altered in neutralization by these mAbs. As part of this experiment, the same strains of RRV were passaged in mosquito cells to determine whether genetic changes were selected in E2 by growth of RRV in mosquito cells and to compare any amino acid changes with those found in RRV passaged in human and avian cells.

This chapter describes the passaging of the NB5092 and T48 strains of RRV in three human cell lines; SW13, 293 and HeLa and in the C6/36 *Aedes albopictus* mosquito cell line. Neither strain of RRV was isolated from humans and they have not been passaged in human cell lines in the laboratory (although both have been plaque purified in an African green monkey cell line). The passaged virus populations were not plaque purified but were subjected to antigenic and sequence analysis to determine whether alterations had occurred in E2 on passaging. Initially only the region around the defined neutralization epitopes (amino acids 216-251) was sequenced, however since no alterations were found in this area, sequence analysis was extended to cover the 5' three-quarters of the E2 gene and in several cases the entire E2 gene. Sequence alterations were analysed in terms of their location in the E2 glycoprotein and changes to hydropathy and predicted secondary structure.

5.2 Materials and Methods

5.2.1 Virus stocks

The plaque purified clones of RRV T48 and NB5092 used in this work were as described in Chapter 4.

5.2.2 Cell lines

SW13 cells, a continuous human adenocarcinoma line were provided by Dr. J.H. Strauss (California Institute of Technology, Pasadena, USA). 293 cells, a human foetal kidney cell line transformed with the E1A gene of adenovirus and HeLa cells, a continuous human adenocarcinoma line, were provided by Dr. A. Bellett (John Curtin School of Medical Research, Canberra, Australia). All human cell lines were grown at 36°/5% CO_2, in EMEM plus 10% FCS. The propagation of mosquito cells (C6/36, *Aedes albopictus*) has been described in Chapter 3. The growth of Vero cell monolayers was as described in Chapter 2.

5.2.3 Antiserum

Monoclonal antibodies used in these studies were as described in Chapters 2 and 4.

5.2.4 Plaque assays

Plaque assays were performed on Vero cell monolayers as described in Chapter 2.

5.2.5 Passaging RRV in human and arthropod cells

Confluent human cell monolayers in 35mm dishes were infected with clones 1/0, 2/0 or 3/0 of either RRV T48 or NB5092 at a moi of 0.5-1. The

monolayers were incubated for the development of cpe, at which time the culture medium was harvested; if cpe was not apparent the medium was harvested after 48 hours. 100µl of the culture medium was used to infect the next monolayer in the series for a total of 10 passages.

For passaging RRV in mosquito cells an initial low moi (~0.01), of the virus clones described above, was used (see Results). The mosquito cell line used in these passaging experiments does not develop cpe when infected with RRV and the culture medium was harvested after 24 hours. 100µl of the first passage supernatant was used as the inoculum at the second passage and then 100µl of a 10^{-2} dilution of the preceding culture medium was used as the inoculum in subsequent passages. A total of five passages was performed in mosquito cells. For each cell line, virus obtained from the final passage was titrated on Vero cell monolayers and used as a working stock for subsequent experiments.

5.2 6 Extraction of infected cell RNA

Infected cell RNA was extracted from the cell line in which the passaging had been done infected with the appropriate working stock, as described in Chapter 3.

5.2.7 Nucleotide sequencing reactions using infected cell RNA

Sequencing reactions and electrophoresis were as described in Chapter 3. The following primers were used:

6K 2347, GGTTTTGTTCTCGTC; E2 1987, GATGATGCGCTCAGA;

E2 1632, TTGTACCTGATAGTC; E2 1452, ACTTCTCTCTACCCACC;

E2 1225, GGGCGTGGGTACCTG. Primer DNA sequences are written in the 5'-3' direction. Priming sites are numbered from the 5' end of RRV NB5092 26S RNA (Faragher *et al.*,1988). The sequences of unpassaged T48 and NB5092 were obtained in parallel using IC/RNA from BHK cells. Where crossbanding could not be resolved but the pattern was identical to that of

the unpassaged virus it was assumed that no nucleotide change had occurred.

5.2.8 Plaque reduction neutralization assays

PRNA on Vero cell monolayers were as described in Chapter 2. In all cases non-plaque purified virus was used. The NB5092 working stock from which clones 1/0, 2/0 and 3/0 were obtained was used as a control in all assays as it had previously been demonstrated that the clones did not differ antigenically from each other or the parent stock with these mAbs (see Chapter 4). Similarly the T48 working stock was used as a control for PRNA with passaged T48 after demonstrating that the clones did not differ from each other or the parent stock in neutralization.

5.2.9 Protein structure analysis

The computer analyses of protein secondary structure predictions and hydropathy profiles were done using the PeptideStructure program and presented graphically using PlotStructure (Jameson and Wolf, 1988, Wolf *et al.*,1988) as for Chapter 4.

5.2.10 Virus nomenclature

Virus stocks were named for the strain of RRV used ie T48 or NB, the cell line in which it was passaged, the original clonal stock ie 1, 2 or 3 and the number of passages in that cell line, thus NB-SW13-1/10 represents clone 1 of NB5092 after 10 passages in SW13 cells.

5.3 Results

To determine whether passaging RRV in human or arthropod cells selected for changes in E2, strains T48 and NB5092 were serially passaged in SW13, 293, HeLa and C6/36 cells. Passaging, nucleotide sequence analysis and antigenic analysis are described below for each cell line. Sequence analysis is summarized in Table 5.1.

5.3.1 Passaging RRV NB5092 and T48 in SW13 cells

Three clones of RRV T48 and three clones of RRV NB5092 were each serially passaged in SW13 cells ten times. Virus was harvested according to the appearance of cpe (Methods; 5.2.5). For NB5092 clones cpe took about 24 hours to develop at the first passage this increased to 48 hours until passage 6, when it occurred about 30 hours pi, subsequent passages showed cpe at 24 hours pi. Clones of T48 showed cpe in all cases within 20-24 hours pi. For T48 clones the titres obtained during passaging fell from $2.9\text{-}4.3\text{x}10^7$ at passage 1 to $3.5\text{-}6.1\text{x}10^6$ at passage 6 and $2.3\text{-}3.7\text{x}10^6$ at passage 10 (all titres are expressed in pfu/ml). NB5092 titres were only measured at passage 10 and were $1.6\text{-}2.7\text{x}10^7$ pfu/ml. The observations on cpe suggest that NB5092 decreased in titre during passaging but subsequently returned to a fairly constant titre .

5.3.2 Sequence analysis of the E2 gene of RRV NB5092 and T48 clones passaged 10 times in SW13 cells

To determine whether amino acid changes were selected in E2 during passaging in SW13 cells the complete E2 gene was sequenced for NB-SW13-1/10, 2/10 and 3/10 (Methods; 5.2.7). For NB-SW13-1/10, NB-SW13-2/10 and NB-SW13-3/10, a G→U transversion was found at nucleotide 1,580 (numbering from the 5' end of NB5092 26S RNA, Faragher *et al.*,1988). This caused a codon alteration from CGC→CUC leading to a predicted non-

Table 5.1

Regions examined and amino acid changes and location in the E2 protein of RRV T48 and NB5092 passaged in human and arthropod cells [a]

[a]The RRV NB5092 and T48 passage populations sequenced are shown. The regions of E2 sequenced are indicated in terms of the regions of the protein covered. Predicted amino acid changes and position are given; numbering is from the N-terminus of E2. Nil indicates no amino acid change was found; * indicates a change at which the wild type nucleotide was still present in the population. No silent nucleotide changes were found.

Virus	Region of E2 examined	Amino acid changes
NB-SW13-1/10	1-422	Arg178→Leu*
NB-SW13-2/10	1-422	Arg178→Leu*
NB-SW13-3/10	1-422	Arg178→Leu,*
		Asn119→Tyr
T48-SW13-1/10	1-297	nil
T48-SW13-2/10	1-298	nil
T48-SW13-3/10	1-308	Pro162→Ser
NB-293-1/10	1-308	nil
NB-293-2/10	1-308	nil
T48-293-1/10	1-305	Ala11→Thr
T48-293-2/10	1-305	nil
T48-293-3/10	209-306	nil
T48-HeLa-2/10	204-303	nil
T48-HeLa-3/10	220-303	nil
NB-mosq-1/5	1-307	nil
	366-422	
NB-mosq-2/5	1-307	nil
	366-422	
T48-mosq-1/5	1-126	nil
	146-185	
	218-308	
T48-mosq-2/5	1-126	nil
	149-183	
	217-308	

conservative amino acid change (Arg178→Leu) in E2. In all three populations a faint G band could also be seen at this position suggesting that the population was heterogenous at this nucleotide. For NB-SW13-3/10, a second change was found at nucleotide 1,402; an A→U transversion produced a codon alteration from AAU→UAU and a predicted amino acid change (Asn119→Tyr). No other nucleotide changes were found in any of the variants sequenced. Of the three passaged clones, NB-3/10 also retained the strongest G band at nucleotide 1,580. All three clones had previously been sequenced following passage in CEFs (Chapter 4). No changes from the NB5092 sequence were identified at these nucleotides, therefore it is reasonable to assume that the nucleotide changes described occurred during passaging in SW13 cells.

The T48 tenth passage stocks T48-SW13-1/10, T48-SW13-2/10 and T48-SW13-3/10 were sequenced between nucleotides 1,051-1,942, 1,051-1,944 and 1,051-1,976 respectively (numbering from the 5' end of the T48 26S RNA, Dalgarno *et al.*, 1983) and corresponding to amino acids 1-297, 1-298 and 1-308. A single change was found in T48-SW13-3/10 at nucleotide 1,534, a C→U transition causing a codon change of CCC→UCC leading to a predicted, non-conservative, amino acid alteration in E2 (Pro162→Ser). No changes were found in the other clones. To confirm that this change was selected on passaging in SW13 cells, T48-293-3/10 and T48-HeLa-3/10 were sequenced through the region coding for amino acid 162; no change was found from the T48 sequence (data not shown).

5.3.3 PRNA of NB5092 clones following 10 passages in SW13 cells

To determine whether the predicted amino acid changes at position 178 or 119 in NB5092 clones altered neutralization epitopes, antigenic analysis of the NB5092 tenth passaged clones was done using PRNA with a panel of mAbs consisting of T1E7, NB3C4, T10C9 and T1D11 (Table 5.2).

Table 5.2

The neutralization of RRV NB5092 populations, passaged 10 times in SW13 cells, by a panel of mAbs [a]

	Monoclonal antibody			
Virus	T1D11	T1E7	NB3C4	T10C9
NB5092	4.02	4.46	5.96	5.45
NB1/10	3.25	4.48	5.84	5.51
NB2/10	3.38	4.57	5.82	5.48
NB3/10	3.95	4.35	5.88	5.38

[a]Titres shown are the reciprocal of the $\log_{10}$ mAb dilution producing 50% reduction in plaque numbers in a PRNA for NB5092 and the SW13 passaged populations 1/10, 2/10 and 3/10 by mAbs T1D11, T1E7, NB3C4 and T10C9. Ten fold dilution intervals were used in all assays. Differences in titre of less than one dilution interval were not regarded as significant.

There was no significant difference between the neutralization of the NB5092 population and the NB1/10, NB2/10 and NB3/10 populations.

5.3.4 Passaging RRV NB5092 and T48 in 293 cells

Three clones of T48 and two clones of NB5092 (NB1/0 and NB2/0) were serially passaged ten times in 293 cells. T48 clones caused rapid cpe in 293 cells between 18-20 hours post-infection during the first three passages, time to cpe then increased to 27-36 hours from passages 4-8 decreasing to 18 hours at passages 9 and 10. Titres after passage 1 were $1.1\text{-}1.6\text{x}10^7$ pfu/ml, after passage 3, $4.3\text{-}5.5\text{x}10^6$ and after passage 10, $7.2\text{x}10^6\text{-}1.2\text{x}10^7$ pfu/ml. Thus the increased time to show cpe appears to correlate well with a drop in titre during passaging.

When NB5092 clones were passaged in 293 cells cpe was initially observed after 36 hours. No cpe was observed, up to 48 hours, for passages 2 and 3 but for subsequent passages, cpe was consistently observed between 20-24 hours. During passaging titres were $8.0\text{x}10^6$ and $1.1\text{x}10^7$ pfu/ml at passage 1 and 1.3 and $1.8\text{x}10^8$ pfu/ml at passage 10, therefore the decreased time to cpe was associated with an increased moi during passaging.

5.3.5 Sequence analysis of the E2 gene of RRV T48 and NB5092 passaged in 293 cells

The NB5092 passaged viruses NB-293-1/10 and NB-293-2/10 were sequenced between nucleotides 1,048 and 1,958 (numbering from the 5'end of the 26S RNA). This corresponds to amino acids 1-308 of E2. No nucleotide alterations were found in this region for either virus. The T48 passaged clones T48-293-1/10 and T48-293-2/10 were sequenced from nucleotides 1,051-1,965 corresponding to amino acids 1-305; T48-293-3/10 was sequenced between nucleotides 1,679-1,970, which corresponds to amino acids 209-306, this includes the neutralization epitopes described between the RRV E2

glycosylation sites (Vrati *et al.*,1988) A single alteration was found in T48-293-1/10; a G→A transition at nucleotide 1,081 leading to a codon change of GCU→ACU which gives a predicted, non-conservative, amino acid change (Ala11→Thr) in E2. No other nucleotide changes were found.

5.3.6 PRNA of clones of NB5092 and T48 passaged 10 times in 293 cells

To confirm that no changes had been selected in neutralization epitopes during passaging in 293 cells antigenic analysis of the NB5092 passaged virus clones was done using a panel of three mAbs; T1E7, T10C9 and NB3C4 to perform PRNAs (Table 5.3). No significant changes in titre were observed between the tenth passage populations and NB5092. T48-293-1/10 was also assayed against NB3C4, T1D11, T1E7 and T10C9 to determine whether an alteration at amino acid 11 altered neutralization by these mAbs. No differences in titre were found for the passaged populations compared to T48 (data not shown).

5.3.7 Passaging RRV T48 and RRV NB5092 in HeLa cells

Three clones of RRV T48 were passaged serially ten times in HeLa cells; passaging was also attempted with NB5092, however, while NB5092 grew to titres of ~10^7 pfu/ml in the first passage, the virus was consistently lost after 3-4 passages (data not shown). For this reason passaging of NB5092 was not pursued in HeLa cells and only a limited analysis of T48 passaging was undertaken since one of the aims of this work was to compare changes between these two genetic types of RRV.

Titres of T48 clones during passaging were 3.4-4.1x10^6 after the first passage and 7.0-9.4x10^6 pfu/ml after the tenth passage. Unequivocal cpe was not observed until passage 4 and after this, consistently occurred 22-36 hours post-infection. Thus while T48 also does not appear to have grown particularly well in HeLa cells there is evidence of some adaptation to this

Table 5.3

The neutralization of RRV NB5092 populations, passaged 10 times in 293 cells, by a panel of mAbs [a]

	Monoclonal antibody		
Virus	T1E7	NB3C4	T10C9
NB5092	4.34 (4.34)	5.51 (5.65)	4.82
1/10	5.36 (5.12)	6.00 (5.86)	5.26
2/10	5.07 (4.70)	6.00 (nd)	4.82

[a]Titres shown are the reciprocal of the $\log_{10}$ mAb dilution producing 50% reduction in plaque numbers in a PRNA for NB5092 and the 293 passaged populations NB1/10 and NB2/10 by mAbs T1E7, NB3C4 and T10C9. Ten fold dilution intervals were used in all assays; values in brackets represent repeat determinations. Differences in titre of less than one dilution interval were not regarded as significant.

nd not determined

cell line during passaging. T48 clones 1/10 and 2/10 were sequenced between nucleotides 1664-1958 and 1710-1958 respectively, corresponding to amino acids 204-303 and 220-303. No nucleotide alterations were found in the regions sequenced. No antigenic analysis of the T48 passaged clones was done.

5.3.8 Growth and passaging of RRV T48 and RRV NB5092 in arthropod cells

Three clones of RRV T48 and NB5092 were passaged five times in C6/36 (*Aedes albopictus*) cells. The passaging protocol used in this cell line differed from the previously used serial passaging by incorporating a low moi first passage. This was to enable growth of first passage virus that would have no or minimal genetic alterations but would be changed in the phenotypic characters typical of mosquito cell grown virus ie membrane composition and lack of sialic acid in added carbohydrate chains (Stollar *et al.*,1976; Luukkonen *et al.*,1977; Hsieh and Robbins, 1984). It was of interest to examine whether this effect alone could be distinguished, in virus grown in mosquito cells, using mAbs with epitopes around the glycosylation sites on the E2 glycoprotein. The second intention of this work was to examine the E2 glycoprotein for genetic changes following passage in mosquito cells. Because of the high titres ($>10^8$ pfu/ml) obtained at each passage level the later passages were made at a 10^{-2} dilution to minimize the potential for interference.

5.3.9 Antigenic analysis of RRV T48 and RRV NB5092 passaged in C6/36 cells

PRNA using mAbs T1E7, T10C9 and NB3C4 with T48-1/5, T48-2/5, T48-3/5 and NB-1/5 and NB-1/1 showed no difference in neutralization titres compared with the unpassaged controls (Table 5.4). This implied that neutralizing antigenic sites associated with these three mAbs were not

Table 5.4

The neutralization of RRV NB5092 and T48 populations, by a panel of mAbs, following passaging in mosquito cells [a]

Virus	Monoclonal antibody		
	T1E7	NB3C4	T10C9
T48	5.30	5.72	5.76
T48-1/5	5.37	5.74	5.92
T48-2/5	5.30	5.62	5.35
T48-3/5	4.97	5.56	5.35
NB5092	4.91	6.23	5.07 (5.22)
NB-1/1	4.54	6.04	5.21 (5.43)
NB-1/5	5.07	6.17	5.53 (5.53)

[a]Titres shown are the reciprocal of the $\log_{10}$ mAb dilution producing 50% reduction in plaque numbers in a PRNA for RRV T48 and NB5092 and the mosquito cell passaged populations T48-1/5, T48-2/5, T48-3/5, NB-1/1 and NB-1/5. The T48 and NB passage series were assayed on separate occasions and the results are shown together for convenience, therefore no comparison can be made between NB5092 and T48 neutralization titres. Ten fold dilution intervals were used throughout; repeat determinations are shown in brackets. Differences in titre of less than one dilution interval were not regarded as significant.

altered either phenotypically by growth of the virus in mosquito cells or genetically by limited passaging in mosquito cells.

5.3.10 Sequence analysis of the E2 gene of RRV T48 and RRV NB5092 passaged in C6/36 cells

Nucleotide sequence was obtained for NB-mosq-1/5 and NB-mosq-2/5. The region sequenced was from nucleotides 1,048-1,973 and from 2,145-2,330 corresponding to amino acids 1-307 and 366-422 of E2. No nucleotide changes were found. For T48-mosq-1/5 and T48-mosq-2/5, nucleotides 1,051-1,427, corresponding to amino acids 1-126; nucleotides 1,488-1,603 and 1,496-1,600, corresponding to amino acids 146-185 and 149-183; and nucleotides 1,706-1,975 and 1,704-1,975, corresponding to amino acids 218-308 and 217-308 were sequenced. No nucleotide alterations were found in either of these mosquito-cell passaged populations. Although there are gaps in this sequence the regions in which changes had previously been found in other passage series are covered.

5.3.11 Analysis of amino acid changes in terms of amino acid sequence conservation, hydropathy profiles and predicted protein structure

The passaging done, the cell lines used, regions of E2 sequenced, and predicted amino acid alterations are summarized in Table 5.1. The majority of changes in E2 were found in virus clones passaged in SW13 cells. A consistent alteration of arginine to leucine at amino acid 178 was found for all three NB5092 passaged populations.

The arginine at position 178 is conserved between RRV and SFV and is in a region of strong amino acid conservation between these two viruses, however, there is virutually no sequence conservation with SIN (Fig.5.1).It might be expected that the virus surface structure could be altered in this region to bury the hydrophobic side chain of leucine in the interior of the

```
NB  E Y Q W G N N P P V R L W A Q L T T E G K P H G W P H E I I  E2 (355)
T48 . . . . . . . . . . . . . . . . . . . . . . . . . . . . .     (355)
SFV . . H . . . . D . . . . . S . . . . . . . . . . . Q . V     (355)
SIN . . I . . . H E . . . V Y . . E S A P . D . . . . . . . V     (356)

NB  Q Y Y Y G L Y P A A T I A A V S G A S L M A L L T L A A T C  E2 (385)
T48 . . . . . . . . . . . . . . . . . . . . . . . . . . . . .     (385)
SFV . . . . . . . . . . . V S . . V . M . . L . . I S I F . S C     (385)
SIN . H . . H R H . V Y . . L . . A S . T V A M M I G V T V A V     (386)

NB  C M L A T A R R K C L T P Y A L T P G A V V P L T L G L L X  E2 (415)
T48 . . . . . . . . . . . . . . . . . . . . . . . . . . . . C     (415)
SFV Y . . V A . . S . . . . . . . . . . . A . . W . . . I . C     (415)
SIN L C A C K . . . E . . . . . . A . N . . I . T S . A . . C     (416)

NB  C A P R A N A                                              E2 (422)
T48 . . . . . . .                                                 (422)
SFV . . . . . H .                                                 (422)
SIN . V R S . . .                                                 (423)
```

A colisting of the amino acid sequence of E2 for RRV NB5092, RRV T48, SFV and SIN. Dots indicate that the residue is identical to the NB5092 sequence, gaps have been introduced to maximize homology. Potential glycosylation sites are underlined. The positions of differences between NB5092 and the human cell passaged variants are indicated (▼). Sequence data is from Dalgarno *et al.* (1983) and Faragher *et al.* (1988).

Figure 5.1

Colisting of RRV NB5092, T48, SFVand SIN E2 amino acid sequences

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                                    ▼
NB  S V I E H F N V Y K A T R P Y L A X C A D C G D G Y F C Y S  E2 (30)
T48 . . T . . . . . . . . . . . . . Y . . . . . . . . . . . .    (30)
SFV . . S Q . . . . . . . . . . I . . . . . . A . H S . H .     (30)
SIN . . . D G . T       L . S . . . G T . S Y . H H T E P . F .  (27)

NB  P V A I E K I R D E A S D G M L K I Q V S A Q I G L D K A G  E2 (60)
T48 . . . . . . . . . . P . . . . . . . . . . . . . . . . . .    (60)
SFV . . . . . A V . S . . T . . . . . . . F . . . . . I . . S D  (60)
SIN . . K . . Q V W . . . D . N T I R . Q T . . . F . Y . Q S .  (57)

NB  T H A H T K M R Y M       A G H D V Q E S K R D S L R V Y T  E2 (87)
T48 . . . . . . I . . .      . . . . . . . . . . . . . . . .    (87)
SFV N . D Y . . I . . A      D . . A I E N A V . S . . K . A .  (87)
SIN A A S A N . Y . . . S L K Q D . T . K . G T M . D I K I S .  (87)

NB  S A A C S I H G T M G H F I V A H C P P G D Y L K X S F E D  E2 (117)
T48 . . . . . . . . . . . . . . . . . . . . . . . V . . . .    (117)
SFV . G D . F V . . . . . . . . L . K . . . . E F . Q V . I Q .  (117)
SIN . G P . A A L S Y K . Y . L . . K . . . . . S V T V . I V S  (117)
      ▼
NB  A N S H V K A C K V Q Y K H D P L P V G R E K F V V R P H F  E2 (147)
T48 . D . . . . . . . . . . . . . . . . . . . . . . . . . . .    (147)
SFV T R N A . R . . R I . . H . . . Q . . . . . . . T I . . . Y  (147)
SIN S   N S A T S . T L A R . I K . K F . . . . . Y D L P . V H  (146)
                                    ▼
NB  G V E L P C T S Y Q L T T A P T D E E I D M H T P P D I P D  E2 (177)
T48 . . . . . . . . . . . . . . . . . . . . . . . . . . . .    (177)
SFV . K . I . . . T . . Q . . . E . V . . . . . . M . . . T . .  (177)
SIN . K K I . . . V . D R L K E T . A G Y . T . . R . R P H A Y  (176)
    ▼
NB  R T L L S Q T A G N V   K I T A G G R T I R Y N C T C G R D  E2 (206)
T48 . . . . . . . . . . .   . . . . . . . . . . . . . . . .    (206)
SFV . . . . . Q S . . . .   . . . V . . K.K.V K . . . . . T G  (206)
SIN T S Y . E E S S . K . Y A K P P S . . N . T . E . K . . D Y  (206)

NB  N V G T T S T D K T I N T C   K I D Q C H A A V T S H D K W  E2 (235)
T48 . . . . . . . . . . . . .    . . . . . . . . . . . . . .    (235)
SFV . . . . . N S . M . . . . .  L . E . . . V S . . D . K . .  (235)
SIN K T . . V . . R T E . T G . T A . K . . V . Y K S D Q T . .  (236)

NB  X F T S P F V P R A D Q T A R K G K V H V P F P L T N V T C  E2 (265)
T48 Q . . . . . . . . . . . . . R . . . . . . . . . . . . .    (265)
SFV Q . N . . . . . . . E P . . . . . . . I . . . . D . I . .  (265)
SIN V . N . . D L I . H . D H T A Q . . L . L . . K . I P S . .  (266)

NB  R V P L A R A P D V T Y G K K E V T L R L H P D H P T X F S  E2 (295)
T48 . . . . . . . . . . . . . . . . . . . . . . . . . L . .    (295)
SFV . . . M . . E . T . I H . . R . . . . H . . . . . . L . .  (295)
SIN M . . V . H . . N . I H . F . H I S . Q . D T . . L . L L T  (296)

NB  Y R S L G A V P H P Y E E W V D K F S E R I I P V T E E G I  E2 (325)
T48 . . . . . . E . . . . . . . . . . . . . . . . . . . . .    (325)
SFV . . T . . E D . Q Y H . . . . T A A V . . T . . . P V D . M  (325)
SIN T . R . . . N . E . T T . . I V G K T V . N F T . D R D . L  (326)
```

protein. This was examined using hydropathy profiles generated for the passaged virus and NB5092 E2 sequence. The change from an arginine to a leucine altered a hydrophilic region to a strongly hydrophobic peak (Fig 5.2). There was no change in predicted secondary structure using either the Chou and Fasman or Garnier algorithm, although, the two methods do not agree on the predicted structure of this region (Fig. 5.2). NB-SW13-3/10 is also altered at a second residue Asn119→Tyr. This is in a region of poorly conserved amino acid sequence and the amino acid at this position is not conserved between NB5092 and T48 (Fig 5.1). This residue is in a moderately hydrophilic part of the protein in a region of predicted alpha helix. The Asn→Tyr change reduces hydrophilicity slightly but does not change the predicted secondary structure (data not shown).

For T48 passaged in SW13 cells one amino acid alteration was found in one passage series (SW13-3/10 Pro162→Ser). This residue of E2 is not strongly conserved but is within a region of amino acid sequence conservation between RRV and SFV (Fig 5.1). The change from a proline might be expected to alter predicted secondary structure but this amino acid is in a short unstructured region of the protein and no change was observed in predicted secondary structure or in hydropathy (data not shown). Only one other change in E2 was detected in these passaging experiments (T48-293-1/10; Ala11→Thr). The amino acid sequence at this position is conserved between RRV and SFV but not SIN. This is in a hydrophilic region of E2 and the change to a polar amino acid increased the hydrophilicity of the region marginally. The Chou-Fasman algorithm predicted that amino acid 11 was at the end of a weak helix forming region which was abolished by the amino acid change, however, the Garnier model predicted a segment of turns and random coil, which was not changed by the Ala→Thr substitution.

In summary, based on hydrophobicity plots, changes in amino acid sequence occurred at positions that were likely to be exposed on the surface of the protein. None of the changes occurred in the region of known neutralization epitopes on E2 nor did these changes alter the antigenic reactivity of the passaged populations.

5.4 Discussion

5.4.1 Amino acid changes selected in E2 during passaging in human cell lines

Non-conservative amino acid alterations were selected in the E2 glycoprotein on passaging clones of RRV NB5092 and T48 in human cell lines. Most of the changes occurred in SW13 cells. In all three passage series of NB5092 in SW13 cells a change at Arg178→Leu was selected. The NB-SW13-3/10 passage population had two predicted amino acid sequence changes in E2; the change at position 178 described above and in addition a change at position 119 (Asn→Tyr). The nucleotide at position 1,580 (amino acid 178 codon) was heterogenous on sequencing gels but there was no evidence of heterogeneity at nucleotide 1,480 (amino acid 119 codon) suggesting that this change was selected early in the passaging series before the change at nucleotide 1,580. Amino acid 178 is in a region of E2 which is strongly conserved between RRV and SFV. T48 passaged in SW13 cells did not alter in this region, however, a change did occur in one T48 passage series (T48-SW13-3/10) at amino acid 162 (Pro→Ser). This residue is in a highly conserved region of E2 but the amino acid at 162 is not conserved. This may imply that the residue is not critical in terms of protein structure and function and therefore that alteration at this position

Figure 5.2

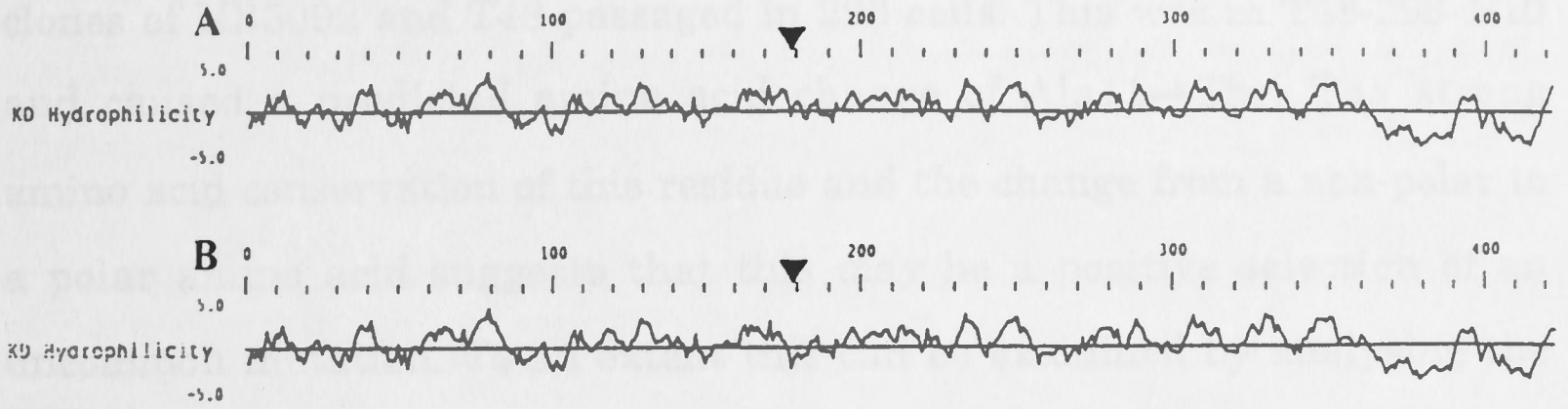
Hydropathy profiles and secondary structure predictions for the E2 glycoprotein of RRV NB5092 and RRV NB-SW13-1/10

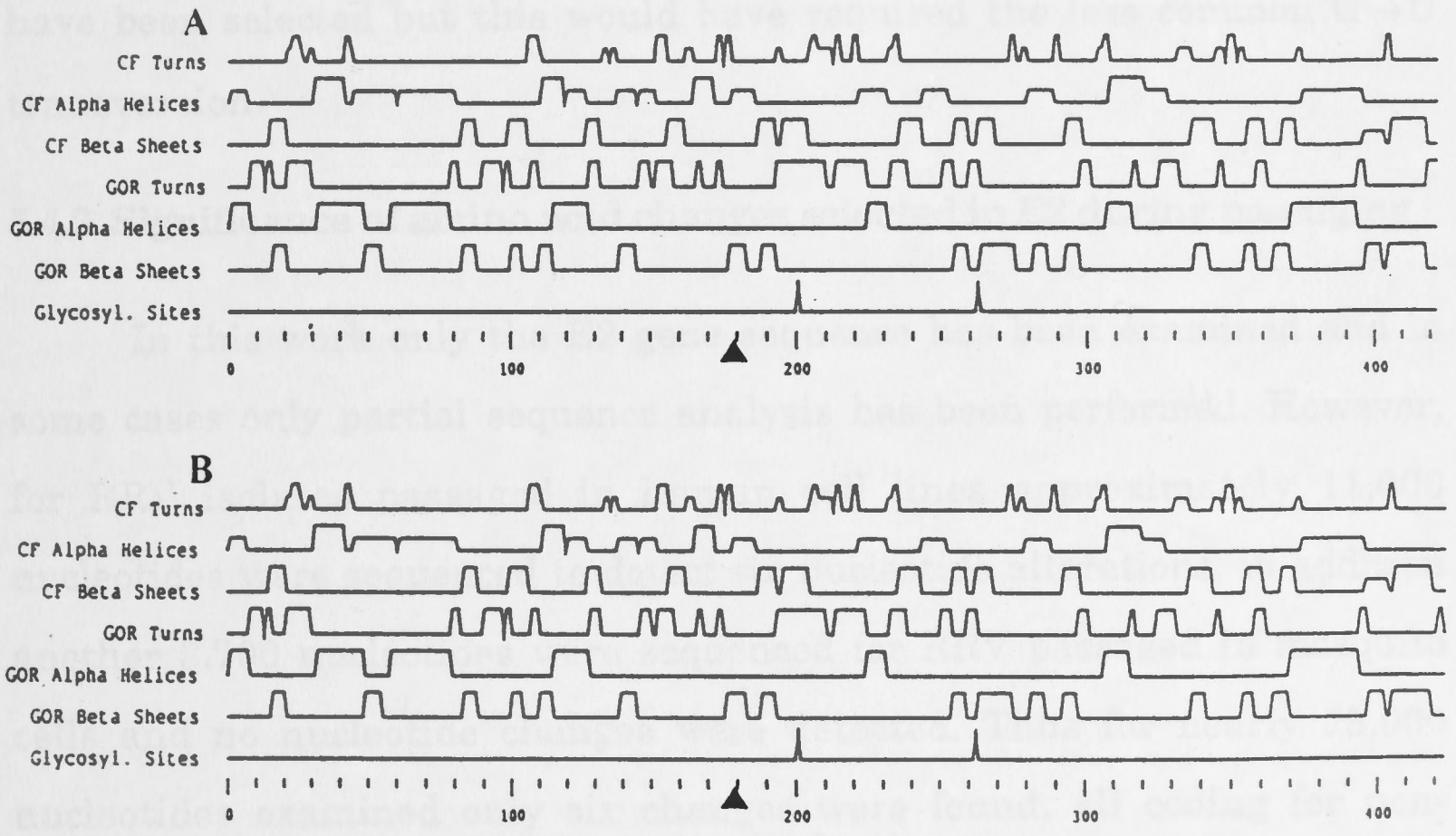
Analysis of protein hydropathy profiles and secondary structure predictions were done using the PeptideStructure program and presented graphically using PlotStructure (Jameson and Wolf, 1988; Wolf *et al.*,1988). These programs use the method of Kyte and Doolittle (1982) for prediction of hydropathy (indicated as KD). Note that values are multiplied by -1 so that hydrophilicity increases with distance above the axis and hydrophobicity increases with distance below the axis. Modified algorithms of Chou and Fasman (1978) and Garnier *et al.* (1978) are used for prediction of secondary structure (indicated as CF and GOR respectively). Glycosylation sites are indicated beneath the secondary structure predictions.

A NB5092

B NB-SW13-1/10

▼ indicates position 178


A
0
100
200
300
400
5.0
KD Hydrophilicity
-5.0
B
0
100
200
300
400
5.0
KD Hydrophilicity
-5.0


A
CF Turns
CF Alpha Helices
CF Beta Sheets
GOR Turns
GOR Alpha Helices
GOR Beta Sheets
Glycosyl. Sites
0
100
200
300
400
B
CF Turns
CF Alpha Helices
CF Beta Sheets
GOR Turns
GOR Alpha Helices
GOR Beta Sheets
Glycosyl. Sites
0
100
200
300
400

may be neutral. In support of this the alteration of Pro162→Ser did not change the predicted secondary structure of the protein.

Only one nucleotide change, in one passage series, was found in clones of NB5092 and T48 passaged in 293 cells. This was in T48-293-1/10 and caused a predicted amino acid change of Ala11→Thr. The strong amino acid conservation of this residue and the change from a non-polar to a polar amino acid suggests that this may be a positive selection of an uncommon mutation. To an extent this can be examined by analysing the possible single base mutations in this codon. Any third position change is silent and only the observed G→A transition leads to Thr. Serine could also have been selected but this would have required the less common G→U transversion.

5.4.2 Significance of amino acid changes selected in E2 during passaging

In this work only the E2 gene sequence has been examined and in some cases only partial sequence analysis has been performed. However, for RRV isolates passaged in human cell lines approximately 11,000 nucleotides were sequenced to detect six nucleotide alterations, in addition another 3,700 nucleotides were sequenced for RRV passaged in mosquito cells and no nucleotide changes were detected. Thus for nearly 15,000 nucleotides examined only six changes were found, all coding for non-conservative amino acid alterations. For CEF passaged clones of NB5092 (see Chapter 4) approximately 7,500 nucleotides were sequenced in E1 and E2 to define three nucleotide changes in E2, again all leading to non-conservative amino acid alterations. Thus a total of approximately 22 kilobases was sequenced to define nine nucleotide changes none of which were silent and all of which coded for non-conservative amino acid substitutions. As discussed in Chapter 4 this suggests that amino acid changes at specific sites are the results of positive selection during

passaging. But the possibility of neutral mutations which do not affect protein stucture and function cannot be excluded. The change at position 162 of T48 passaged in SW13 cells is a possible example of such a mutation as it is an amino acid which is not conserved between T48 and NB5092 and occurs in a region of the protein which is predicted to be relatively unstructured.

One aim of this work was to compare the amino acid changes selected in E2 for two strains of RRV on passaging in human and arthropod cells with those selected in NB5092 passaged in avian cells. None of the changes selected on passaging in human cells were at the same amino acid position as the alterations selected in CEFs. Nor were changes to positively charged amino acids selected. In addition the neutralization of human passaged variants was not altered by the panel of mAbs tested. As the amino acid changes in CEF passaged NB5092 occurred in or around neutralization epitopes it seems that these changes may not be closely related to those selected during human cell passaging. This is exemplified by the changes in the N-terminal portion of E2: an amino acid change at residue 4 (Glu→Lys) of NB5092 altered neutralization by mAbs NB3C4 and T1D11 (see Chapter 4), however, a change at residue 11 (Ala→Ser) of T48 did not change neutralization by these mAbs.

The T48 and NB5092 strains of RRV did not have the same amino acid changes selected on passaging in human cell lines. In the primary amino acid sequence of E2 the changes at 178 and 162 are quite close but it is unknown how these residues relate in the folded protein. It appears that changes selected during cell passaging of RRV were both cell and virus strain specific.

The functional significance of those amino acid changes selected during passaging is unknown because, apart from neutralization assays,

no experiments have been done to examine altered phenotypic properties of the passaged virus populations. The changes may represent adaptations at different phases of the virus replication cycle. These include cell attachment, receptor binding, virus uncoating, intracellular processing and transportation or virus assembly and release. They may in some cases be selected under the pressures that develop during passaging that are virus rather than cell driven, for example, interfering activity. The simplest explanation is that these changes are an adaptation to a specific cell type although the adaptative value of changes in E2 may not be immediately apparent from simple sequence inspection. It is interesting to speculate that the Arg→Leu alteration at 178, which causes a significant increase in hydrophobicity in this region of the protein and occurred in all three passage series may have been associated with the change in growth characteristics of NB5092 clones in SW13 cells which was observed as a decreased time to cpe.

Further studies would be required to define the adaptive significance of the amino acid changes which have been identified in E2 following passaging in human cells. These types of studies include plaque purification of virus from different passage levels and examination of virus isolates for phenotypic alterations as well as attempting to define genetic changes in other regions of the genome. Phenotypic studies such as *in vivo* and *in vitro* growth of virus, growth in different cell lines and RNA synthesis kinetics would all be useful in defining the mechanism of selection of such changes and helping to determine whether non-structural proteins or non-coding regions of the genome were involved. Further development of a cell binding assay for RRV would allow study of cell attachment and internalization kinetics of RRV variants.

5.4.3 Conservation of antigenic properties and genetic sequence of RRV following growth and passaging in mosquito cells

Passaging viruses in mosquito cells could alter antigenic reactivity by either selected genetic changes or by host cell derived phenotypic changes to the carbohydrate moieties at the E2 glycosylation sites. The neutralization of RRV NB5092 and T48, by a panel of neutralizing mAbs with epitopes close to the E2 glycosylation sites in the primary polypeptide sequence, was not affected by growth or passaging in mosquito cells compared with unpassaged virus grown in mammalian cells. It appears that the addition of sialic acid and consequent alteration in charge and hydrophilicity of these regions of E2 is not important in terms of antibody binding and neutralization.

Two strains of RRV passaged in mosquito cells showed no alterations in nucleotide sequence in E2. This may indicate that for RRV there is little selective pressure for amino acid changes in E2 in these cells. This is in contrast to the situation with RRV strains passaged in avian or human cell lines and also to the situation in some Bunyaviruses passaged in mosquito cells (James and Millican, 1986). Mutations in E2 selected on passaging alphaviruses in mosquito cells can affect virus assembly. Durbin and Stollar (1986) found mutations in the E2 gene of a strain of SIN following passage in mosquito cell culture. These mutations were associated with a range of phenotypic characteristics including host cell restriction. Amino acid differences to the parental virus were at positions 55, 172 and 275, with the change at 275 introducing a new glycosylation site. Revertants selected by passage in CEFs had lost this glycosylation site which appeared to interfere with virus assembly in vertebrate cells. The changes at 55 and 172 did not revert.

Chapter 6

General Discussion

For RRV the three defined neutralization epitopes (a, b and c) appear to cluster into a major neutralization domain on E2. Sites at the N-terminus of the protein, and around amino acids 55-61 (Vrati *et al.*,1986) possibly fold into apposition with sites at 232-234 (epitope b1) and are closely associated with epitope b2 (246, 248, 251). Monoclonal antibodies to epitope b compete with mAbs to epitope a at 216 (Vrati *et al.*,1988) and this epitope is also affected by changes at amino acid 218, (but not 219, Burness *et al.*, 1988). Epitope c is defined by mAb T1D11, which competes with mAbs defining epitope b (Vrati *et al.*,1988) and is at least partially defined by amino acid 4 on E2.

Amino acid changes in epitope b2 increased the rate of penetration of RRV T48 suggesting that conformational changes of a broad nature in this epitope impinge on a site involved in cell penetration. Passaging RRV NB5092 in avian cells selected variants which were altered in epitopes a and the combination of b and c as judged by decreases in neutralization titres of mAbs. Both sets of variants were somewhat altered in their pattern of mouse growth compared with the parental NB5092 but those altered at epitope a were attenuated for growth in mice. This suggested that changes in these epitopes were involved in adaptation to new cell lines and may also be involved in virus growth in mice, perhaps by influencing tissue tropism. Changes that were selected in E2 on passaging in human cell lines did not occur in known neutralization epitopes. Other phenotypic properties of variants selected in human cell passaging have not been examined.

Meek *et al.* (1989) showed that variants of RRV NB5092 selected by passaging in mice had alterations in the E2 protein. Five of eight variants had E2 alterations at amino acids 212, 232, 234 and 251, within, or close to, neutralization epitopes. These changes were associated with increased

virulence in mice but were not the primary cause of this. It was postulated that they represented an adaptation to growth in mouse tissues.

The only other alphavirus for which neutralization epitopes have been mapped by selection of mAb escape mutants is SIN. Three epitopes have been defined on E2; a, 190, b, 216 and c, 62, 96, 159 (Davis *et al.*,1987; Strauss *et al.*,1987; Pence *et al.*,1990). On the basis of CBAs, epitopes E2a and E2b appear to lie in relatively close proximity to each other while epitope E2c is spatially separate (Olmsted *et al.*,1986). This epitope defines functional domains on E2 of SIN associated with penetration and neurovirulence that interact in a complex manner as shown by selection of escape mutants (Pence *et al.*,1990). An equivalent epitope has not been defined on RRV E2, although epitope b2 shares some of these properties as discussed in Chapter 3.

E2 is the most variable of the alphavirus structural proteins (Levinson *et al.*,1990). This can be correlated with its role as the protein to which the majority of neutralizing antibodies are directed (Dalrymple *et al.*,1976) and its position as probably the more exposed protein on the viral surface (Sefton *et al.*,1973; Dalrymple *et al.*,1976). It has been suggested that amino acid changes in alphavirus structural proteins may be immunologically selected (Levinson *et al.*,1990), however, there are several lines of evidence that suggest that antibodies may not be a major selective pressure on RRV under natural conditions. Firstly few changes were selected in neutralization epitopes during divergence of RRV strains NB5092 and T48 (see Chapter 2). Similarly during a major epidemic of RRV in the Pacific islands involving tens of thousands of infections only one amino acid change was found in E2, and although this was close to epitope a, in the primary sequence, it did not alter neutralization with a panel of mAbs (Burness *et al.*,1988). These authors suggested that this low rate of mutation, compared with non-arboviruses, could be because

transmission to mosquitoes is likely to occur predominantly at the time of peak viraemia, whereas antigenic variants, selected under the pressure of antibodies, are likely to arise later during infection when successful transmission to mosquitoes is less probable because of the lower viraemia. In support of this, the work presented in Chapter 2 demonstrated that several amino acid alterations, at specific sites, were necessary for RRV T48 to acquire substantial resistance to polyclonal neutralization, especially during the primary immune response. Finally, passaging in tissue culture selected variants that had changes in neutralization epitopes on E2. As antibody was unlikely to have been involved in such selection it appears that variations in E2 neutralization epitopes were selected as an adaptation to host or growth conditions rather than by antigenic pressure.

Several well characterized viral systems can be compared with RRV. The haemagglutinin (HA) of influenza A and B viruses is a homodimer of HA1 and HA2 which exists as trimeric spikes inserted in the virion membrane (Wiley and Skehel, 1987). The structure of this protein has been extensively studied and it provides a useful paradigm for virus surface proteins. Like the surface spikes of RRV this molecule functions in cell attachment, entry and fusion and is the protein to which neutralizing antibodies are directed. Unlike RRV E2 it appears to undergo significant antigenic drift due to immune pressure. A large number of neutralizing antigenic sites have been mapped onto the three dimensional structure of this protein using mAb escape mutants and natural isolates. Most of the variation is in the HA1 protein, involving residues covering the distal surface of the molecule (Wiley and Skehel, 1987). HA1 also contains the receptor binding site, which is a conserved pocket in the distal portion of the molecule surrounded by a group of antibody binding sites. Because of the proximity of epitopes to the receptor binding pocket, certain amino acid

substitutions can prevent neutralization by mAbs, alter receptor specificity and affinity and may be associated with changes in tissue tropism and virulence *in vivo* (Deom *et al.*,1986; Wiley and Skehel, 1987; Philpott *et al.*,1990).

For example escape mutants of a highly virulent avian influenza strain with single amino acid changes, within an epitope located near the distal tip of the HA molecule and close to the receptor binding site, were attenuated in chickens compared to the parental virus and appeared to have altered tissue tropism as the virus no longer replicated in splenic macrophages (Philpott *et al.*,1990). Thus attenuation of this virus probably resulted from changes in tissue tropism brought about by alterations in receptor affinity. This is in contrast to other attenuating mutations of avian influenza which involved alterations in the cleavage site of the HA molecule such that HA0 was no longer efficiently cleaved to HA1 and HA2 by host cell proteases (Webster and Rott, 1987). Other workers have demonstrated that addition of an oligosaccharide chain close to the receptor binding pocket can reduce affinity for mammalian host cells and change antigenicity. Virus with this mutation was selected against during passage in bovine kidney cells while in CEFs both parental and mutant viruses grew equally well (Crecelius *et al.*,1984; Deom *et al.*,1986).

Another example is poliovirus which contains a major antigenic loop to which neutralizing antibodies are directed (antigenic site 1) which is formed by a continuous sequence of amino acids 90-105 in VP1 (Hogle *et al.*,1985). This domain is also an important determinant of host range for poliovirus. When this region of the mouse avirulent Mahoney strain of poliovirus 1 was replaced with the homologous region of the mouse adapted Lansing strain of poliovirus 2, the hybrid virus induced paralytic disease in mice (Murray *et al.*,1988). This demonstrated that a short sequence of amino acids in antigenic site 1 was responsible for

determining poliovirus host range and suggested that this site may be involved in attachment of poliovirus to cells of the mouse central nervous system. It has been proposed that an analogous site on VP1 is involved in cell attachment of FMDV (Acharya *et al.*,1989).

Similarly neutralizing mAb escape mutants of human reovirus were attenuated in mice and restricted in their distribution in mouse brain compared to the parental virus suggesting that these neutralization epitopes defined regions involved in tissue tropism (Spriggs and Field, 1982). It is clear that in a number of viruses there is a subset of neutralization epitopes clustered around domains involved in cell attachment and that definition of these neutralization sites has increased understanding of the interaction of the virus with host cells.

From the evidence discussed, it seems reasonable to postulate that the cluster of neutralization epitopes a, b and c may interact in a domain on RRV E2 that mediates cell penetration, adaptation to novel cell types and growth *in vivo*. This is consistent with other viruses such as influenza, poliovirus and FMDV, where groups of neutralization epitopes cluster around or partially define domains involved in receptor recognition, tissue tropism and virulence. The value of such a model lies in focussing attention on this region of RRV and for developing further experiments which may define the structure and functions of this region of RRV more adequately.

Such experiments would probably develop along three lines using techniques from immunology, molecular biology and cell biology. In the first instance the selection of further neutralizing mAbs could help to define functional domains on E2 and possibly E1 by selection of escape mutants. By analogy with the E2c epitope of SIN (Olmsted *at al.*,1984), selection of mAbs using variants of RRV that are altered in different

regions of E2, especially regions which alter functions or which are predicted to alter the local conformation of the protein, could provide a useful range of antibodies. For example NB1/5ws altered at amino acid 4 and NB-SW13 variants changed at residue 178 could be used to produce mAbs which could in turn allow selection of a range of escape mutants.

By using transcribable, complete cDNA clones of RRV specific changes in E2 could be made to test the functional relationships of alterations such as those at amino acid 218 to growth in mice. Preliminary constructs have already been prepared with this change but have not been tested in mice (J. Strauss, R. C. Weir and L. Dalgarno, pers. comm.). This technique provides a powerful tool for investigating the functions of RRV genes, proteins and regulatory regions and experiments such as those described in this thesis provide a starting point for studies using transcribable cDNA clones.

Much can be done at the level of cell biology to investigate the interactions of RRV with cultured cells. Preliminary work on the development of a cell binding assay for RRV has been described in Chapter 3. Further work should be done to examine the rate of binding of fast penetrating variants of T48 and other isolates of RRV. It would be extremely interesting to select fast penetrating variants of RRV using the selection protocol of Olmsted *et al.* (1984), where virus was allowed to adsorb to cells for 60 min at 4°, shifted to 37° for 1 min and unpenetrated virus was removed from the cell surface by protease digestion. Variants of both SIN and VEE selected for fast penetration using this protocol are also reduced in virulence in mice. In SIN such fast penetration mutants are changed in reactivity at neutralization epitope E2c. Fast penetrating variants of RRV selected using this protocol could then be compared with the fast penetrating variants of T48, altered at epitope b2 and with the

geographic isolates of RRV to further define regions of E2 involved in fast penetration and to explore the relationship of penetration and virulence.

Ultimately, with an infectious clone of RRV, it may be possible to test specific mutations of E2 which prevent attachment of the virus to certain cell types or to all cell types and map regions involved in attachment to cells and thus receptor recognition.

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